Genome-wide Screens to Identify Novel Components of the Metal Response in *Drosophila melanogaster*

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2006

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Summary

All organisms have to ensure metal homeostasis by coping with fluctuating amounts of trace elements in the environment. Genetic and biochemical studies in mammals and *Drosophila* have established a central role for metal-responsive transcription factor MTF-1 in transition metal homeostasis. MTF-1 is an essential gene in mammals. In *Drosophila,* MTF-1 is not required for viability. However, in the fly, as in mammals, it is a critical factor for metal tolerance, and, unexpectedly, also for the copper starvation response. Even though great progress in the biology of transition metals has been made, there is still a lack of knowledge about the pathways and mechanisms leading to metal response and the genes involved in the immediate handling of various metals.

In this study we attempted to identify novel components of the metal response using microarray based transcriptome analysis and genome-wide RNA interference technique in *Drosophila melanogaster*.

Firstly, the transcriptome of MTF-1 mutant and wild type larvae, raised in normal or metal-supplemented food, was compared. This revealed new candidate MTF-1 target genes, such as the copper importer *Ctr1B*, the zinc exporter *ZnT-D1*, the ABC transporter *CG10505,* and the ferritin genes *Fer1HCH* and *Fer2LCH*. Further biochemical and genetic analyses in the fly established these candidates as genuine MTF-1 regulated genes and explained various aspects of *Drosophila* MTF-1 knockout phenotype. Moreover, we have uncovered a variety of genes that respond to metal load, independent of MTF-1. Among these are the genes involved in the glutathione-mediated detoxification pathway, and the genes of heat shock and immune responses.

In the second analysis a genome-wide RNAi screen was done in cultured *Drosophila* S2 cells in collaboration with the RNAi Screening Center at Harvard Medical School. The screen identified a large number of candidate genes acting upstream of the promoter of metallothionein A, one of the best characterized target genes of MTF-1. A secondary screen using in house RNAi procedures was performed to validate the data. Several candidate genes appear to be involved in a pathway that regulates the metallothionein promoter. Future studies are needed for a detailed characterization of these genes and for placing them within the hierarchy of the metal stress response pathway.

Zusammenfassung

Alle Organismen müssen Metallhomöostase sicherstellen, indem sie fluktuierende Mengen von Spurenelementen in der Umwelt bewältigen. Genetische und biochemische Studien in Säuger und Fliegen zeigten eine zentrale Rolle für den metalloregulatorische Protein MTF-1 (metal-responsive transcription factor) in der Homöostase von Übergangsmetallen. MTF-1 ist ein essentielles Gen in Säugern, währenddem es in *Drosophila* nicht überlebenswichtig ist. In beiden Fällen aber, in der Fliege und in Säugern, ist es ein kritischer Faktor für die Toleranz gegenüber Metallen und in *Drosophila,* unerwarteterweise auch gegenüber Kupfermangel. Obwohl grosse Fortschritte in der Biologie der Übergangsmetallen gemacht worden sind, bleibt das Wissen über Signalwege und Mechanismen der Antwort auf Metallstress lückenhaft. Auch ist wenig bekannt über die Gene, die in der unmittelbaren Handhabung von verschiedenen Metallen involviert sind.

In dieser Studie versuchten wir, neue Komponenten der Antwort auf Metallstress in *Drosophila melanogaster* mit Hilfe einer Microarray basierten Transkriptom Analyse und genomweiter RNA Interferenz zu identifizieren.

Zuerst wurde das Transkriptom von MTF-1 mutanten und wildtyp Larven verglichen. Beide Genotypen wurden in normalem Futter und solchem mit Metallzusätzen aufgezogen. Dadurch wurden neue, mögliche MTF-1 Zielgene gefunden, wie z.B. der Kupferimporter *Ctr1B*, der Zinkexporter *ZnT-D1*, der ABC Transporter *CG10505* und die Ferritin-Gene *Fer1HCH* und *Fer2LCH*. Weitere biochemische und genetische Analysen in der Fliege bestätigten diese Kandidaten als MTF-1 Zielgene und erklärten einige Aspekte des *Drosophila* MTF-1 knockout Phänotyps. Ausserdem entdeckten wir eine Anzahl von Genen, die unabhängig von MTF-1 auf Metallzusatz reagierten. Unter diesen sind die Gene des Glutathion basierten Detoxifikation Signalwegs und die Gene involviert in Hitzeschock und Immunantwort.

In der zweiten Analyse wurde ein genomweiter RNAi Screen in *Drosophila* S2 Zellkulturen durchgeführt, in Zusammenarbeit mit dem RNAi Screening Center an der Harvard Medical School. Der Screen identifizierte eine grosse Anzahl von Kandidatengenen, welche den Promotor vom Metallothionein A (*MtnA*) regulieren. *MtnA*

ist eines der am Besten charakterisierten Zielgenen von MTF-1. Ein zweiter Screen mit hausinternen RNAi Verfahren wurde durchgeführt, um die Daten zu bestätigen. Einige Kandidatengene scheinen in einen Signalweg involviert zu sein, der den MtnA promotor reguliert. Weitere Studien sind notwendig für eine detaillierte Charakterisierung dieser Gene, und um ihren Platz in der Hierarchie der Antwort auf Metallstress zu bestimmen.

General Introduction

Every living organism has to ensure the right amounts of nutritional elements for proper growth, development and metabolic maintenance. Transition metals (hereafter referred to also as heavy metals) are among these essential elements. They are needed in a great variety of biochemical processes: respiration, signal transduction, gene expression, detoxification, neuropeptide production - to list only few. Many essential metals are scarce in the environment and the organisms have developed specific mechanisms for their efficient acquisition and retention. On the other hand, if the amounts of the metal ions are exceeding a certain threshold, they may harm the cells and the very function they serve. Excess metal can bind to inappropriate sites in enzymes and thus interfere with their functions. Redox active metals such as copper or iron can catalyze a Fenton reaction resulting in hydroxyl radical production, the most aggressive of all reactive oxygen species (ROS). Thus organisms have to protect themselves also against the deleterious effects of metal load. The cell metal concentration can fluctuate beyond the physiological limits if the metal homeostasis mechanisms are compromised (e.g., due to genetic disorders) or due to large changes in the environment (e.g. metal pollution). This can lead to various diseases and syndromes and even to death. The heavy metals copper, zinc and cadmium and syndromes associated with abnormal amounts of each are introduced below.

To maintain the physiological, relatively stable concentrations of metals organisms have developed specific homeostatic mechanisms: metal import, sequestration, donation of metal ions, and metal efflux. Accordingly, molecules and regulatory mechanisms have been evolved to carry out each function at the right position and timely fashion. Metal transporters, metallochaperones, sequestration molecules and the regulatory factors involved in metal homeostasis are described later.

Even though great progress in the understanding of biological roles of transition metals has been made, there is still a lack of knowledge about the pathways and mechanisms leading to metal response, and it seems safe to say that many metal transporters and molecules involved in metal response await characterization. Genome-wide screenings have proven to be powerful tools for the identification of new molecules and regulatory mechanisms. Transcriptome analysis using microarray

technology and genome-wide RNA interference are the major methods used in this study for the identification of new components of the metal response.

Drosophila melanogaster is a convenient system for the study of metal homeostasis since many aspects of metal response and regulation are conserved in this genetically easily tractable model organism. A great advantage is also the availability of genome sequences of at least seven species of the *Drosophila* genus.

Biology and toxicity of transition metals copper, zinc and cadmium

Copper

Copper is an essential element for living systems, as it is required for the catalytic activity of a number of essential enzymes (Table 1). Formation of active copper-dependent enzymes takes place with participation of auxiliary proteins, so called copper-chaperones (see below) that deliver copper ions to these enzymes.

Table 1

Copper containing enzymes	biological process
cytochrome C oxidase	electron transport, ATP generation
Cu-Zn superoxide dismutase (SOD)	radical scavenging
angiogenin	blood vessel formation
Fet3	iron uptake
ceruloplasmin	iron homeostasis
hephaestin	iron transport
dopamine β-hydroxylase	catecholamine production
peptidoglycine α-hydroxylating monooxygenase	bioactivation of peptide hormones
tyrosinase	melanin production
lysyl oxidase	extracellular matrix protein cross-linking

In the cell, copper ions can be in the Cu (I) or Cu (II) state. This valency change is crucial for copper function as a cofactor in the active centers of some enzymes such as cytochrome C oxidase in the respiratory chain. But the same redox potential renders copper detrimental if this metal is in excess: it catalyzes the synthesis of reactive oxygen species (ROS) via the so-called Fenton reaction. ROS can cause serious damage to lipids, proteins and nucleic acids.

Copper is primarily absorbed in the small intestine. The copper ATPases ATP7A and ATP7B, which are affected in the genetic disorders of Menkes and Wilson's diseases, respectively, are key molecules to regulate the flow of copper (Bull et al. 1993; Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). It is believed that ATP7A exports copper from the enterocytes, supplying the organism with this essential metal. ATP7B is expressed in hepatocytes and in response to copper overload it exports excess copper into the bile. Mutations in ATP7A cause a genetic disorder, Menkes disease, a copper deficiency syndrome which can lead to growth retardation, white, kinky hair and mental deterioration. The genetic disorder Wilson's disease, a copper toxication syndrome manifested by liver damage, is caused by a malfunction of ATP7B. Copper exposure (derived from milk or water kept in copper vessels) can cause Indian childhood cirrhosis, which leads to hepatic copper overload and serious liver damage (Tanner 1998).

Apart from the specific inborn disorders of copper, evidence is growing that copper is involved in common neurologic conditions, such as Alzheimer's disease, Parkinson's disease, the prion diseases, and amyotrophic lateral sclerosis (Sayre et al. 2000; Valentine et al. 2004).

Zinc

Zinc is an essential catalytic and structural component of more than 300 enzymes (Eide 2003). It is not redox-active under physiological conditions and this is probably the reason why it is considerably less toxic than copper. However, at high concentrations zinc may bind to inappropriate sites in proteins or cofactors and interfere with their functions. Zinc toxicity has been found to be associated with reduced iron absorption, impaired immune function (Whittaker 1998) and neuronal death (Koh and Choi 1994; Chen and Liao 2003; Chen and Liao 2003). The uptake and efflux of zinc ions is mediated by a number of zinc transporters. Mutations in some of these zinc transporters have been found to be associated with zinc metabolism disorders. The ZIP4 zinc importer is associated with the genetic zinc deficiency disorder acrodermatitis enteropathica that is characterized by dermatological lesions, impaired wound healing, immune and reproductive problems and lack of weight gain during development (Dufner-Beattie et al. 2003). Zinc deficiency may play a role also in the cause of ADHD (attention deficit hyperactivity

disorder) in children (Sandyk 1990). Mutations in murine zinc exporter ZnT-4 are responsible for lethal milk (lm) mouse syndrome (Huang and Gitschier 1997).

Cadmium

Cadmium is a non-essential heavy metal. It is a significant environmental pollutant and is very toxic even at low concentrations. Humans are exposed to cadmium through contaminated food, water, air and especially cigarette smoke. Cd^{2+} exists as a stable cation in the cell and does not undergo redox reactions. Nonetheless, it depletes antioxidant components, notably glutathione and protein-bound sulfhydryl groups, thus causing enhanced production of reactive oxygen species (Stohs et al. 2000). The production of ROS and radicals has been proposed as a mechanism for acute cadmium toxicity. Cadmium can replace other metals, notably zinc and iron, and can also substitute for calcium in bone (Ando et al. 1978; Wang and Bhattacharyya 1993). Zinc deficiency is a common cause of cadmium toxicity: when zinc is deficient, the body will absorb cadmium to replace zinc in enzyme binding sites. It has also been reported that cadmium causes apoptosis (Chrestensen et al. 2000), activates the transcription of protooncogenes c-jun, c-fos, c-myc and mdm2 (Jin and Ringertz 1990; Shukla et al. 2000), destabilizes the helical structure of DNA, causes single stranded DNA breaks (Tsuzuki et al. 1994), and, as shown recently, inhibits DNA repair (Jin et al. 2003). Cadmium is classified as a human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Occupational exposure to it has been associated with cancers of the lung, the prostate, the pancreas, and the kidney (for review see (Waalkes 2003)). In the last decade studies have shown that cadmium can act like steroidal estrogen in breast cancer cells as a result of its ability to form a high-affinity complex with the hormone binding domain of the estrogen receptor (Garcia-Morales et al. 1994; Stoica et al. 2000). Recently the estrogen-like activity of cadmium was shown *in vivo* (Johnson et al. 2003). Cadmium exposure can lead to renal dysfunction, anemia, chronic rhinitis, osteoporosis, and bone fractures (reviewed in (Jarup et al. 1998)). A classical example of industrial cadmium pollution was the outbreak of Itai-Itai disease in Japan several decades ago with symptoms of severe pain, bone fractures, proteinuria and severe osteomalacia (Jarup 2002).

Regulation of metal homeostasis

Metal homeostasis is the process that maintains the equilibrium of the cell's and the organism's metal concentrations. This maintenance happens at the level of the metal uptake, sequestration, distribution and efflux.

Metal transporters

Metal ion transporters play a major role in the metal homeostasis of the cell and the organism. Metal importers provide efficient metal uptake from limited environmental sources whereas the exporters fence off an excess of metal from the cytoplasm. During the last decade numerous studies conducted from yeast to mammals led to a detailed understanding of metal transport and its regulation. The orthologs of several metal transporters have been found and characterized also in *Drosophila*. Separate high-affinity systems for copper, zinc, iron, and manganese are responsible for providing to the cell the particular element when it is in short supply. In addition, low-affinity systems play a "housekeeping" role, supplying a specific metal when it is more abundant in the environment. Many metal transporters are mono-specific, while some can mediate the transport of a variety of metal ions.

Copper transporters. As mentioned above, Two membrane-bound P-type ATPase enzymes, ATP7A and ATP7B, the products of genes involved in Menkes and Wilson disease, respectively, are responsible for cellular copper efflux in mammals (Fig.1). ATP7A and ATP7B have one common ortholog in *Drosophila*, DmATP7. The knock-down of *DmATP7* in Schneider S2 cells via

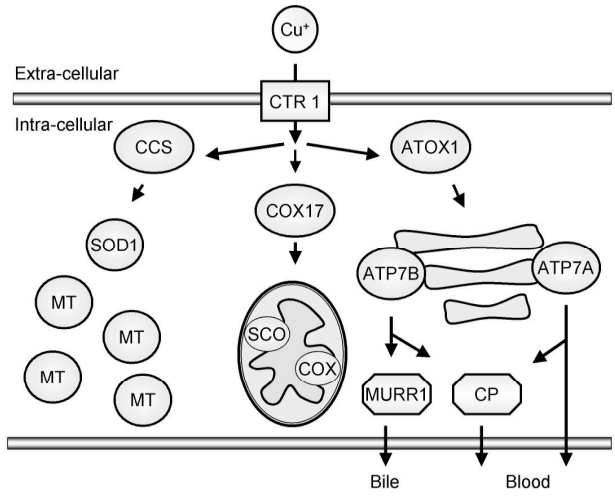

Figure 1: Schematic overview of intracellular copper trafficking in hepatocytes.

Adapted from (Spee et al. 2005)

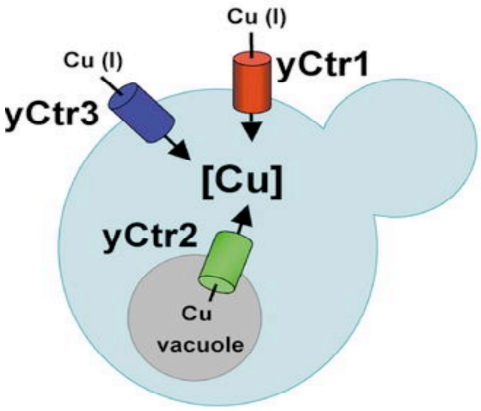

Figure 2: The location of Ctr transporters in *S. cerevisiae*

Adapted from MJ Petris, 2004

RNA interference dramatically increases copper accumulation (Southon et al. 2004).

A class of transporters known as the SLC31 (solute-linked carrier 31) or Ctr (copper transporter) family of proteins mediates cellular copper uptake. Several Ctr genes are characterized in different organisms, such as Ctr1, Ctr3 (high affinity copper transport systems) and Ctr2 (a low affinity copper transport system) in yeast (Dancis et al. 1994) (Fig.2), COPT1 in Arabidopsis (Kampfenkel et al. 1995) or hCtr1 (Zhou and Gitschier 1997; Moller et al. 2000) in humans. Three high affinity copper importers, Ctr1A, Ctr1B and Ctr1C, are known in *Drosophila melanogaster* (Zhou et al. 2003). A null mutant for *Drosophila* Ctr1B has been generated, which is viable under the normal experimental conditions but extremely sensitive to nutritional copper scarcity and, to a lesser degree, also to copper load. Ctr1B mutant animals show decreased larval copper accumulation and abdominal pigmentation defects due to insufficient tyrosinase activity (Zhou et al. 2003).

Zinc transporters. Zinc transport is mediated via two families of SLC proteins:

a) SLC30, also called CDF (cation diffusion facilitator) or ZnT (Zn transporters). The members of this family reduce intracellular cytoplasmic zinc concentrations by promoting zinc efflux from cells to the exterior or into intracellular vesicles.
b) SLC39, also called ZIP (zinc-regulated transporter and iron-regulated transporter like proteins). The proteins in this family function in the uptake of zinc to the cytoplasm.

There are more than 100 ZnT and 86 ZIP transporters known in organisms of all phylogenetic levels (for review see (Liuzzi and Cousins 2004). The number of known mammalian zinc transporters has increased dramatically in recent years. Nine ZnT proteins (ZnT1-to-ZnT9) and at least seven ZIP proteins (ZIP1-to-ZIP4, LIV-1,

BIGM103, hKE4) are characterized or predicted in mammals. Figure 3 summarizes the present knowledge on subcellular localization and zinc ion/ hormone regulation of several mammalian zinc transporters. More than ten zinc transporter genes are annotated in *Drosophila melanogaster* (FlyBase) based only on sequence similarities to vertebrate zinc transporters. None of them has been characterized so far.

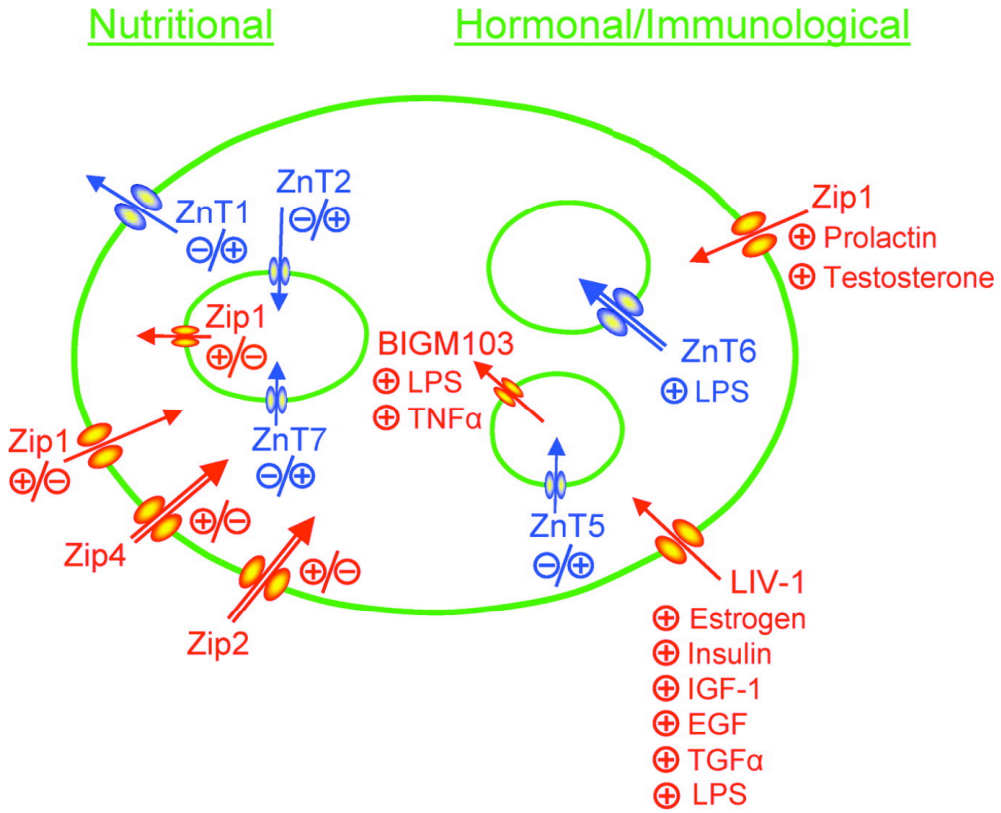

Figure 3: Some of the observed nutritional and physiological inputs that influence expression of mammalian zinc transporters. Nutritional regulation is shown as the direction of response to zinc depletion/supplementation (i.e.,-/+ or +/-). Physiologic regulation shows responses in integrative or isolated cell systems (illustrated only as a positive response).

Abbreviations: EGF, epidermal growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor

Adapted from Liuzzi and Cousins, 2004

Iron transporters. Divalent metal transporter 1 (DMT1, also called DCT1, Nramp2 or SLC11A2) is responsible for transferrin-independent uptake of dietary iron from

the intestinal lumen (for review of iron transport see (Wessling-Resnick 2000)). DMT1 is involved also in manganese and cobalt transport, and probably in the uptake of other metals. Expression studies with Xenopus oocytes or in mammalian cells suggest that DMT1 is also importing cadmium (Picard et al. 2000; Okubo et al. 2003). Nramp1 (natural resistance-associated macrophage protein 1) is a close paralog of Nramp2/DMT1 and, may also import Fe^{2+}, Mn^{2+} and Co^{2}. Transferrin (Tf)/ Transferrin receptor (TfR) system, as well as HFE protein which interacts with TfR, regulate intestinal iron absorption. SFT (stimulator of iron transport) is another transporter involved in iron uptake. In yeast, a low-affinity transporter FET4 is responsible for the uptake in iron replete cells. High-affinity transporters FET3 and FTR1 are induced in iron-depleted cells. Two proteins, FTH1 and FET5, are localized at the vacuolar surface and are responsible for mobilizing intravacuolar iron stores (Urbanowski and Piper 1999). Metal transporter protein 1 (MTP-1, also known as ferroportin 1, Ireg1, or SLC11A3) is an iron exporting protein. The expression of MTP-1 is induced upon iron exposure (Yang et al. 2002). Yet another gene involved in iron transport especially from mitochondria, is frataxin. Mutations in the frataxin gene (especially an expansion of GAA triplets in the first intron) are responsible for Friedreich's ataxia, the most common of the inherited ataxias (reviewed in (Pandolfo 2001). Several observations reinforce the hypothesis that an altered iron metabolism, free radical damage, and mitochondrial dysfunction all occur in Friedreich ataxia.

So far only one iron transporter of relatively broad metal specificity was found in *Drosophila*. It is the product of malvolio (mvl) gene. Mvl mutant *Drosophila* have defects in tasting behaviour, which can be restored by addition of Fe or Mn salts to the food (Rodrigues et al. 1995; Orgad et al. 1997). Mvl shares 65% identity to mammalian Nramp1.

Table 2 summarizes the current knowledge on major mammalian metal transporters and the associated diseases.

Table 2

Major copper, zinc and iron transporters and diseases associated with their misregulation.

Name	Metal transported	Function/expression	Disease
MNK	Cu2+	Basolateral exit; intestine	Menkes disease
WND	Cu2+	Exit, biliary excretion	Wilson's disease
hCTR1	Cu2+	Uptake	-
hCTR2	Cu2+	n.d.	-
ZnT-1	Zn2+	Basolateral exit	-
ZnT-2	Zn2+	Vacuolar exit	-
ZnT-3	Zn2+	Synaptic vesicles	-
ZnT-4	Zn2+	Export/lactation	Lethal milk (mouse)
ZnT-5	Zn2+	Export: pancreas, ovary, prostate, testis (hs)	- -
ZnT-6	Zn2+	Export: liver, brain, intestine	-
ZnT-7	Zn2+	Transport into vesicles, small intestine, liver	-
Zip1	Zn2+	Uptake: intestine	-
Zip2	Zn2+	Uptake: prostate, uterus	-
Zip3	Zn2+	Uptake: bone marrow, spleen	-
Zip4	Zn2+	Uptake: small intestine, kidney	Acrodermatitis enteropathica
DCT1/Nramp2	Fe2+, Co2+, Mn2+,	Uptake/endosomal exit	Hemochromatosis, microcytic anaemia other?
Nramp1	Fe2+, Mn2+, other?	Phagosom/lysosomal exit	Infectious susceptibility
SFT	Fe2+/Fe3+	Uptake	-
HFE	Fe	Regulation of intestinal absorption	Hereditary hemochromatosis (HH) type 1
Tf/TfR system	Fer2+	Uptake	Hypotransferrin-emia (Tf), HH type 3 (TfR2)
MTP-1	Fe	Export: intestine, lungs etc.	HH type 4
Frataxin/FRDA	Fe2+/Fe3+	Mitochondrial export	Friedreich's ataxia

Metallochaperones

The assembly of the metal centers in various enzymes is a complex process, involving many accessory or helper proteins. Metallochaperones comprise a class of proteins which bind metal ions and deliver them directly to target enzymes via protein-protein interactions. Current knowledge of metallochaperones comes predominantly from the extensive studies of copper chaperones. Proteins involved in nickel (Colpas et al. 1999), iron (Nguyen et al. 1999), and molybdenum (Leimkuhler and Klipp 1999) cofactor delivery and insertion have also been identified.

Three types of copper chaperones have been found in eukaryotes (see Fig.1):

1. Copper chaperones for copper-transporting ATPases: Atx1 in yeast, Hah1, Atox1 in mammals, Cch in plants, CopZ in bacteria.
2. Copper chaperone for SOD1: yCCS in yeast, hCCS in humans
3. Copper chaperones for cytochrome C oxidase: Cox17, Cox11, Sco1, Sco2.

These proteins have been characterized from yeast to mammals by the elucidation of their three-dimensional structures, intracellular target proteins and the mechanisms of action (for review see (Rosenzweig 2002; Markossian and Kurganov 2003)).

The *Drosophila* genome encodes homologs of copper chaperones for superoxide dismutase (CG17753) and for cytochrome C oxidase (CG9065 and CG8885).

Sequestration molecules

An important group of molecules involved in trace element homeostasis and detoxification are sequestration/scavenger/detoxification molecules. They are found in all animal phyla, as well as in plants, fungi and bacteria. The main sequestration molecules including metallothioneins, ferritins, glutathione and phytochelatins, are described below.

Metallothioneins (MTs**)**. MTs are ubiquitous low molecular weight proteins with a high cysteine content (about 30%) and an outstanding capacity to bind heavy metals (for review see (Palmiter 1998)). They have been discovered as a cadmium and zinc containing protein in horse kidney by Margoshes and Vallee in 1957. Although MTs are known for almost half a century, their precise physiological function is still under debate. However, a protective role of MTs against heavy metal exposure and also to oxidative stress has been repeatedly reported in many organisms. Typically, MT genes harbour multiple metal response elements (MREs) in their promoter/enhancer region (described below in the section "metal regulatory factors").

Four metallothionein isoforms are characterized in mammals, MT-1 to MT-4. Under physiological conditions mammalian metallothioneins predominantly bind to zinc. MT-1 and MT-2 are ubiquitously expressed in all mammalian cell types analyzed so far and are strongly induced by heavy metals and some other stress conditions. MT-3 and MT-4 are constitutively expressed and are at most poorly metal-inducible. MT-3, also known as neuronal growth inhibitory factor (GIF), is expressed predominantly in neurons, but also in glia and male reproductive organs (Uchida et al. 1991; Masters et al. 1994; Moffatt and Seguin 1998). MT-4 is expressed in squamous epithelial cells (Quaife et al. 1994). Mice double knockout for MT-1 and MT-2 are viable but considerably more sensitive to cadmium than control mice (Michalska and Choo 1993; Masters et al. 1994; Kelly et al. 1996). The organization of the human metallothionein gene family is complex: hMTs are encoded by a family of genes consisting of ten functional and seven nonfunctional metallothioneins clustered on chromosome 16 (West et al. 1990; Garrett et al. 1998). Baker's yeast *S. cerevisiae* contains a metallothionein (copper-thionein) gene family comprised of the amplified CUP1 locus and the single copy CRS5 gene (Culotta et al. 1994). CUP1 plays the dominant role in copper detoxification, whereby the level of resistance is proportional to the copy number of this locus, which can be found in up

to 15 tandemly iterated copies (Fogel and Welch 1982; Jensen et al. 1996).

The *Drosophila melanogaster* genome encodes four metallothioneins (*MtnA, MtnB, MtnC* and *MtnD*), clustered on chromosome 3 (Lastowski-Perry et al. 1985; Mokdad et al. 1987; Egli et al. 2003). All four *Drosophila* metallothioneins are induced by heavy metals, notably by zinc, cadmium and copper (Zhang et al. 2001; Egli et al. 2003), whereby MtnA and MtnB preferentially confer resistance to copper and cadmium, respectively (Egli et al., submitted). Metallothionein gene duplication has been shown to be coupled to increased metal tolerance in natural populations of *D. melanogaster* (Maroni et al. 1987).

Ferritins are constitutive proteins known to detoxify, store and transport iron. Ferritin in vitro and in vivo binds other metal ions such as Be, Cu, Zn, Cd, Pb and Al. These findings suggest that ferritin may function as a general metal detoxicant (Joshi and Zimmerman 1988).

The vertebrate ferritin is a multimeric protein containing 24 subunits (Andrews et al. 1992). These subunits are divided into two subgroups: heavy and light chains. All currently known insect ferritins can also be divided into heavy-chain homologues (HCH) and light-chain-homologues (LCH), based on similarity to the human heavy- and light-chain ferritin subunits, respectively (Pham et al. 2000). The *Drosophila* genome encodes one gene for each subunit: ferritin 1 heavy chain homolog (Fer1HCH) and ferritin 2 light chain homolog (Fer2LCH) (Dunkov and Georgieva 1999). Transcripts and protein levels for both Fer1HCH and Fer2LCH increase dramatically when larvae are fed a diet rich in iron (Georgieva et al. 1999; Georgieva et al. 2002). Alternative RNA splicing and utilization of different polyadenylation sites were found to generate several ferritin transcripts in *Drosophila* (Lind et al. 1998). The proportion of the mRNAs is strongly influenced by iron feeding (Georgieva et al. 1999; Georgieva et al. 2002).

Glutathione (GSH), a cysteine containing tripeptide (Fig. 4), is a key cellular antioxidant and free radical scavenger (reviewed in (Meister and Anderson 1983)). The expression of proteins involved in GSH biosynthesis and metabolism is induced by cadmium in yeast and mammals (Dormer et al. 2000; Hart et al.

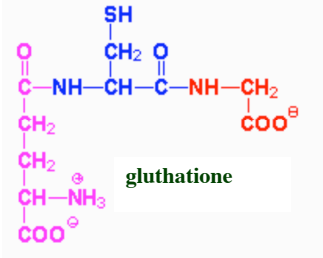

Figure 4: Glutathione: chemical structure

2001; Vido et al. 2001; Wimmer et al. 2005). Many harmful agents, including heavy metals, are scavenged by conjugation to GSH by glutathione S-transferases (GSTs) (Hayes and Pulford 1995). The glutathione conjugates (X-SG) can then be sequestered by the vacuole or transported from the cell by ATP-dependent glutathione S-conjugate efflux pumps (Ishikawa 1992). Of particular interest in this context is the yeast cadmium factor (YCF1), an ATP-binding cassette transporter, which transports cadmium-glutathione conjugates to the vacuole (Fig. 5) and thus protects yeast from cadmium toxicity (Li et al. 1996). The *Drosophila* genome encodes YCF1 homologs and several classes of GSTs but none has been characterized so far.

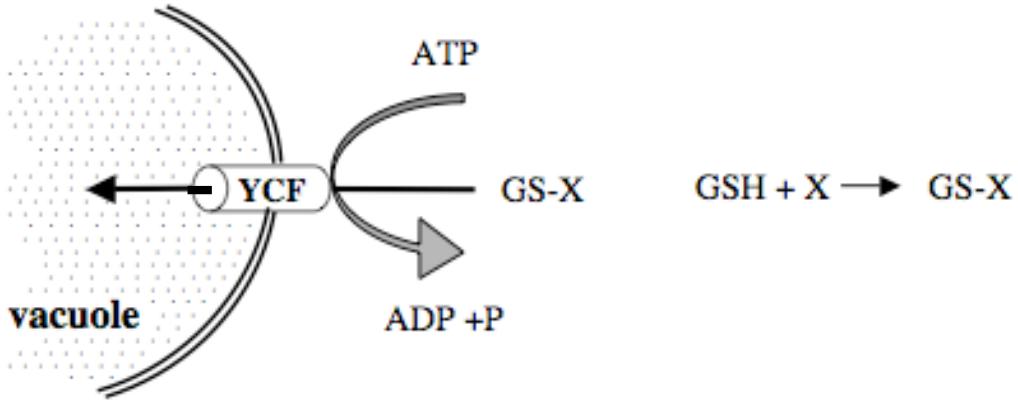

Figure 5
Yeast cadmium factor (YCF), an ATP-binding cassette transporter sequesters glutathione conjugates (GS-X) in the vacuole.

Phytochelatins (PCs) are important heavy metal binding and detoxifying peptides in plants. They are also found in some fungi, for example, in the fission yeast *S. pombe*. PCs are cysteine-containig tripeptides that are enzymatically synthesized from glutathione. In Arabidopsis, Silene, pea and tomato a variety of transition metals (Cd, Ag, Bi, Pb, Zn, Cu, Hg, and Au) induce biosynthesis of phytochelatins (PCs) from GSH (for review see (Cobbett 2000)). Recently PCs have been discovered also in *C. elegans* and shown to be critical for heavy metal tolerance in the worm (Vatamaniuk et al. 2001). Plant PCs and MTs have a great potential as components of detoxification mechanisms used in environmental clean-up, so called phytoremediation, of metal-contaminated areas.

Metal regulatory factors

The regulation of metal homeostasis occurs at gene transcription, translation, message stability, protein translocation and degradation levels. Translational control and RNA stability are the main mechanisms used by iron-regulatory proteins (for review see (Theil and Eisenstein 2000)). Metal-dependent trafficking or degradation is a wide-spread mechanism for regulation of metal transporters (Petris et al. 1996; Kim et al. 2004). Transcriptional regulation plays the major role in the transition metal homeostasis and has been extensively studied from yeast to mammals. The metal transcription factors in fungi and higher eukaryotes are introduced below. Table 3 summarizes the current knowledge about the major metal-responsive transcription factors and their target genes.

Transcriptional regulation of metal homeostasis in fungi

Distinct transcription factors function in yeast to ensure copper, zinc and iron homeostasis. Ace1 in (also known as Cup2) *Sacharomyces cerevisiae* and its functional orthologs Amt1 in *Candida glabrata* and Crf1 *Yarrowia lipolytica* activate gene expression in response to elevated copper (Thiele 1988; Zhou and Thiele 1991; Garcia et al. 2002) while Mac1 *(S. cerevisiae)*, GRISEA (*Podospora anserina*) and Cuf1 (*S. pombe)* activate gene expression in response to copper deficiency (Jungmann et al. 1993; Osiewacz and Nuber 1996). The Zap1 transcription factor from *S. cerevisiae* activates gene expression in response to zinc deficiency (Zhao and Eide 1997). Aft1 and Aft2 activate gene expression in *S. cerevisiae* when iron is scarce. Iron-regulated gene expression in fungi other than *S. cerevisiae* is mediated by a group of GATA-type transcription factors (for a review of metal-responsive transcription factors that regulate Cu, Zn and Fe homeostasis see (Rutherford and Bird 2004)).

Copper resistance in *S. cerevisiae* mediated by Ace1 (activating copper-MT expression) is primarily due to induction of the *CUP1* copper-binding MT gene (Fig. 6A). Ace1 also regulates the expression of a second MT gene (*CRS5*) and the Cu, Zn superoxide dismutase gene (*SOD1*).

Upon copper starvation, Mac1 (metal-binding activator 1) activates the expression of the high-affinity copper uptake systems encoded by *CTR1* and *CTR3*. Mac1 also regulates the expression of a cell surface Fe3+/Cu2+ reductase (*FRE1*) and

a putative reductase *FRE7* (Fig. 6A). The posttranslational degradation of Ctr1 under conditions of excess copper also requires Mac1. In *S. pombe,* in addition to activating copper transporter genes, Cuf1, a functional ortholog of Mac1, represses the expression of the iron-regulated *fip1+*, *fio1+*, and *frp1+* genes that encode proteins required for iron uptake (Labbe et al. 1999). Thus, the ability of Cuf1, and possibly Mac1, to act as both a repressor and activator allows the coordinated expression of genes involved in both copper and iron homeostasis.

Under zinc-limiting conditions, Zap1 (zinc-responsive activator protein 1) increases the expression of zinc uptake systems encoded by the *ZRT1*, *ZRT2*, and *FET4* genes (Fig. 6B, Table 3). Zap1 also stimulates the release of zinc from the vacuolar zinc store by activating the expression of the *ZRT3* vacuolar efflux system (MacDiarmid et al. 2000). Another target of Zap1 is *ZRC1*, a gene that encodes a vacuolar zinc influx system. Although it seems counterintuitive that Zap1 upregulates the expression of a gene that lowers cytoplasmic zinc levels, recent studies have revealed that the increased expression of *ZRC1* in response to zinc limitation is a proactive mechanism to protect zinc-limited cells from possible exposure to high zinc levels, so-called zinc shock (MacDiarmid et al. 2003). In addition to the above-mentioned transporter genes, Zap1 regulates the expression of more than 40 other genes, some of which may have additional roles in zinc homeostasis (Table 3). At the transcriptional level, Zap1 binds to a zinc-responsive element located within its own promoter and autoregulates its own expression (Bird et al. 2000). Zap1 activity is also regulated by several posttranslational mechanisms.

The pair of paralogous iron-responsive transcription activators, Aft1 and Aft2 (activator of ferrous transport) play key roles in the response to a lack of iron in the environment via the induction of genes involved in the transport of iron and its subcellular distribution and use. Aft1 activates the cell surface iron uptake systems upon iron depletion (Fig. 6C, Table 3), Aft2 activates the transcription of genes involved in intracellular iron use in the absence of Aft1 (Table 3). The iron-responsive GATA-type factors repress the transcription of their target genes in response to high iron. Therefore, although the iron-responsive GATA factors are transcriptional repressors and the Aft factors are transcriptional activators, both classes of factors ensure that their target genes are induced when the relevant organism senses that iron is limiting (Rutherford and Bird 2004).

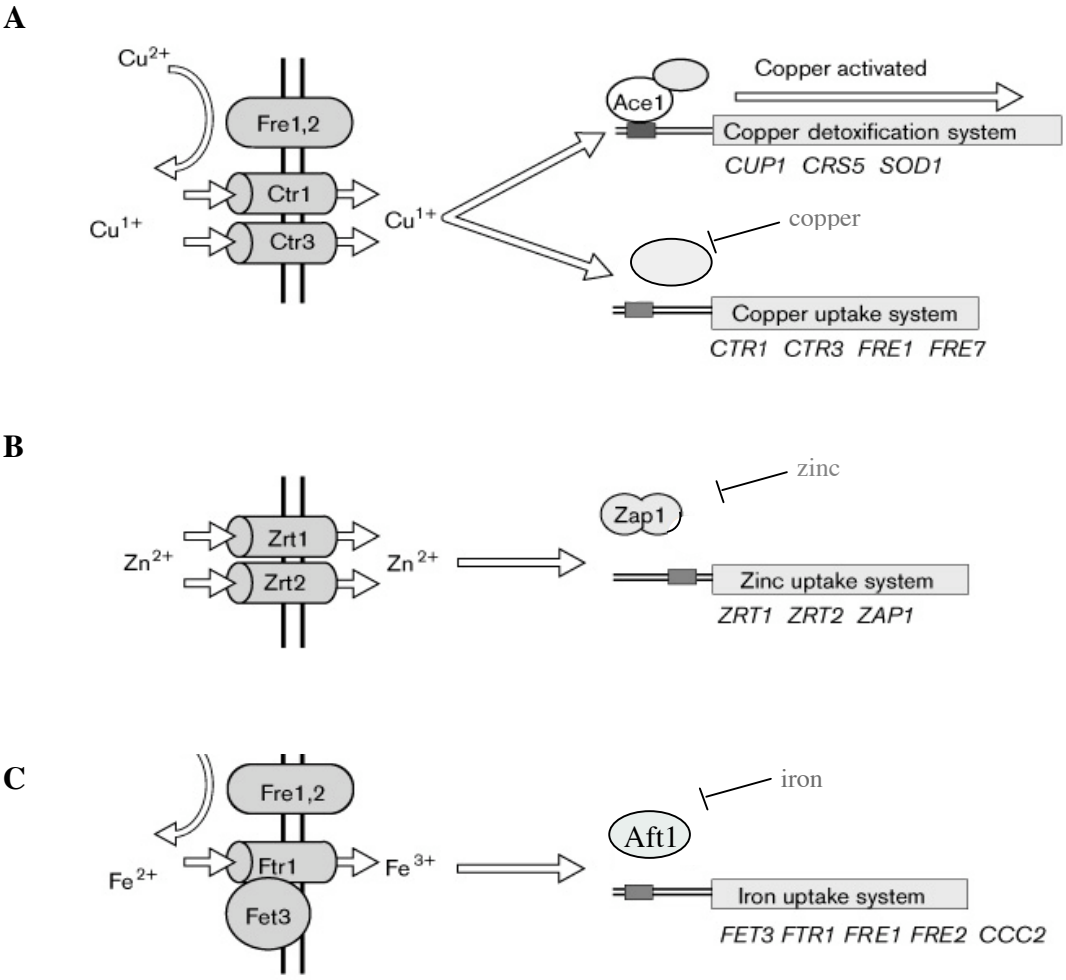

Figure 6

Metalloregulatory pathways in S. cerevisiae. *Modified from Winge et al., 1998*

Transcriptional regulation of metal homeostasis in higher organisms

A key regulator of heavy metal response from insects to mammals is the metal-responsive transcription factor 1 (MTF-1). This is a zinc-finger factor that plays a central role in the transcription of metallothionein genes and other genes involved in stress response. Apart from the zinc-finger domain, MTF-1 has distinct activation domains, namely, an acidic, a proline-rich and a serine/threonine-rich in its C-terminus, that are important for transcriptional activation (Radtke et al. 1995) (Fig. 7).

Recently a cysteine-rich cluster, located close to C-terminus, has been characterized and shown to be crucial for metal-induced transcription (Chen et al. 2004). MTF-1 harbors also a nuclear localization signal towards its N-terminus, and a nuclear export signal within the acidic activation domain.

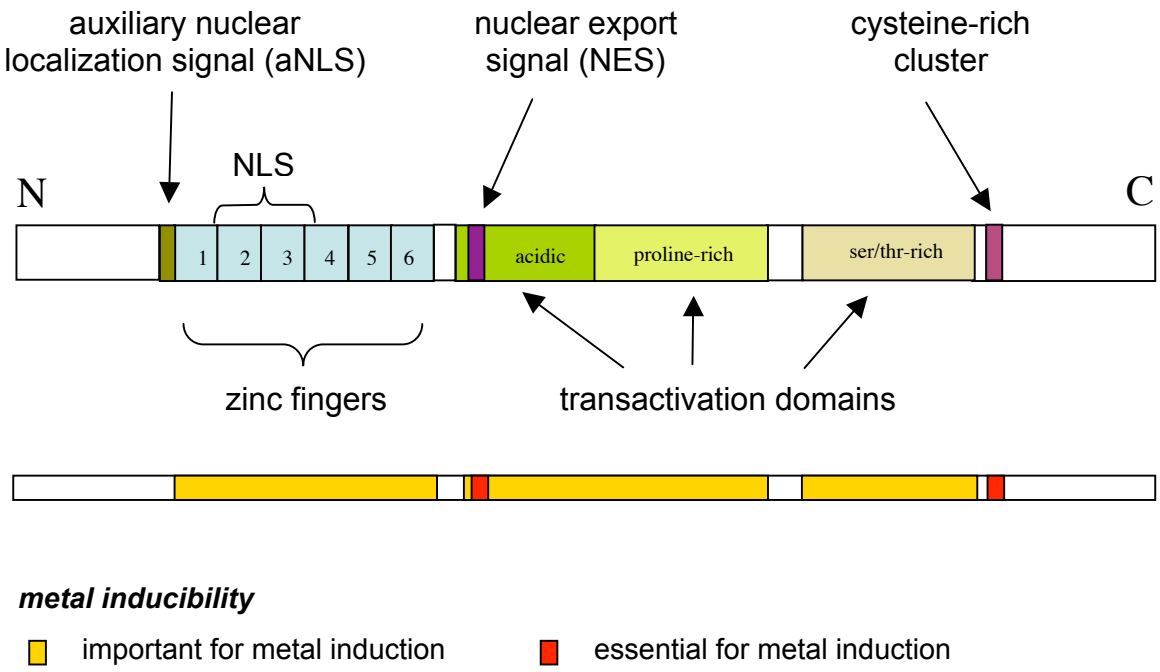

Figure 7

Schematic representation of MTF-1 protein domains and the importance of each in metal inducubility

MTF-1 is mostly cytoplasmic but is translocated to the nucleus upon diverse stress signals, notably by metal load (Smirnova et al. 2000; Saydam et al. 2001). Nucleo-cytoplasmic trafficking of MTF-1 is crucial for its metal induced transcriptional activity (M. Cramer and W. Schaffner, submitted).

MTF-1 has been cloned from humans, mouse, the bony fish *Fugu rubripes, Danio rerio* and the insect *Drosophila melanogaster* (Brugnera et al. 1994; Auf der Maur et al. 1999; Zhang et al. 2001; Chen et al. 2002). The MTF-1 protein sequence

and the functional domains are well conserved between different vertebrate species, but the similarity to the *Drosophila* ortholog is essentially restricted to the zinc finger domain.

MTF-1 mediates transcription via binding of its zinc fingers to metal response elements, so called MREs. MREs cis-acting DNA sequence motifs with a core consensus TGCRCNC (where R stands for adenine or guanine and N for any of the four bases) that are found in the promoter/enhancer regions of all metallothionein genes. The mutation of MRE sequences completely abolishes MT metal induction (Searle et al. 1987); also, MREs alone fused to a minimal promoter confer metal-induced expression of the reporter gene (Stuart et al. 1984; Searle et al. 1985), suggesting that MREs are both necessary and sufficient for heavy metal induced gene expression.

The transcription of MTF-1 itself does not change upon metal load, suggesting a post-translational regulation of metal sensing and activation. The current model of MTF-1 activation derived from in-vitro studies in a cell-free mammalian expression system implies zinc-loaded metallothioneins that release zinc upon cadmium or copper load (Fig. 8). Released zinc then binds and saturates MTF-1 zinc fingers, the protein enters the nucleus and activates transcription via binding to MRE sequences (Zhang et al. 2003).

In addition, it has been shown that MTF-1 is phosphorylated in vivo and this modification plays a critical role in MTF-1 activation by zinc and cadmium. Inhibitor studies indicate that multiple kinases and signal transduction cascades, including those mediated by protein kinase C, tyrosine kinase, and casein kinase II, are essential for zinc- and cadmium-inducible transcriptional activation (Saydam et al. 2002).

In our laboratory knockouts (KO) for mouse and *Drosophila* MTF-1 genes have been generated. In the mouse, disruption of the MTF-1 gene turned out to be an embryonic lethal due to liver degeneration around embryonic day 14 (Gunes et al. 1998). However a conditional knockout in liver and bone marrow, and to a lesser extent in other organs, is viable under non-stress conditions but highly susceptible to cadmium toxicity, in support of a role of MTF-1 in coping with heavy metal stress (Wang et al. 2004). *Drosophila* MTF-1 KO is viable under normal laboratory conditions (Egli et al. 2003). However, it is very sensitive to metal load (zinc, cadmium, copper and mercury), and surprisingly, also to copper depletion.

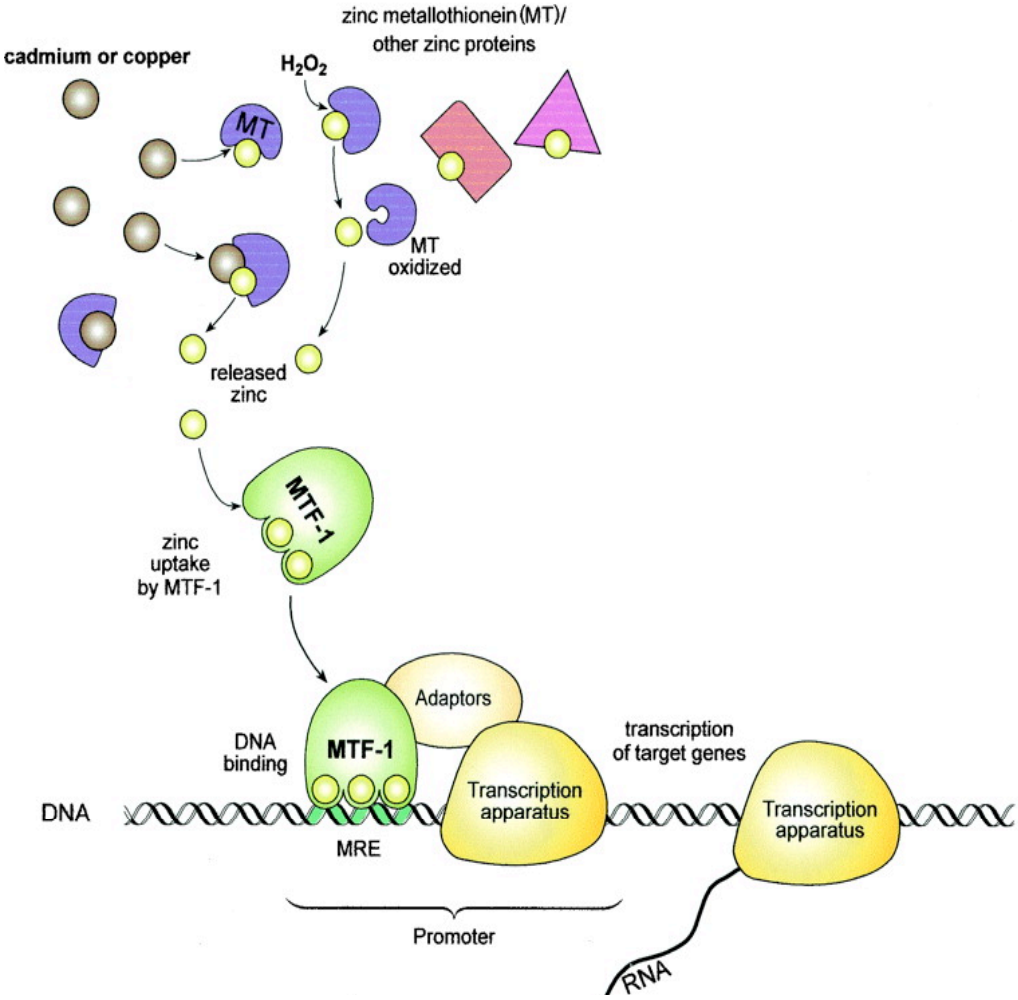

Figure 8

Model for MTF-1 activation by Cd, Cu and H2O2 via Zn-loaded MT. Cd and Cu bind to MT with a much higher affinity than Zn, but due to the great abundance of the latter, the majority of MT is zinc-bound. Upon Cd or Cu loading, Zn is released from MT and presumably from other proteins and allows zinc saturation of MTF-1. H2O2 induces zinc release from MT via oxidation of sulfhydryl groups.

From Zhang et al., 2003

Given the fact the mouse MTF-1 KO is lethal whereas the MT-1/MT-2 double KO is viable, it can be concluded that mMTF-1 activates important genes other than metallothioneins. Transcriptome and other biochemical studies of wt and MTF-1 KO mice suggest several target genes for mMTF-1, besides metallothioneins also a zinc transporter-1 (ZnT-1), tear lipocalin, placenta growth factor (PlGF) etc. (Table 3, for review see (Lichtlen and Schaffner 2001)).

The search and characterization of *Drosophila* MTF-1 target genes is the main part of my thesis project. This study once again confirmed all four *Drosophila* metallothionein genes as dMTF-1 targets and revealed several new genes to be under dMTF-1 control. Some of them are specifically involved in the response to a particular heavy metal, such the copper importer *Ctr1B* that is induced in copper starvation, the zinc exporter *ZnT-D1* (*CG3994*) that is induced by excess zinc and also by cadmium, whereas other candidate dMTF-1 targets, such as the ABC transporter *CG10505,* are affected by all metals tested (zinc, copper and cadmium) (see Results). The null mutants of many dMTF-1 target genes generated and characterized in our laboratory explore different aspects of MTF-1 function in *Drosophila melanogaster*.

Table 3

The listed genes are metal regulated and have a consensus sequence (or sequence that resembles the consensus site) for the relevant transcription factor in the promoter region and/or encode a protein involved in metal metabolism. *Genes repressed by the specified transcription factor

Txn factor	Description	Gene name(s)
Aft1	Transporters	*FET4*, *FET5*, *FTR1*, *FTH1*, *SMF3*, *MRS4*, *CCC2*,*COT1*
	Cu chaperone	*ATX*
	Ferroxidase	*FET3*, *FET5*
	Metalloreductases	*FRE1*, *FRE2*, *FRE3*, *FRE4*, *FRE5*, *FRE6*
	Cell wall proteins	*FIT1*, *FIT2*, *FIT3*
	Siderophore transport	*ARN1*, *ARN2*, *ARN3*, *ARN4*
	Fe-S biosynthesis	*ISU1*, *ISU2*
	Other	TIS11, *HMX1*, *AKR1*, *PCL5*, *YOR387c*, *YHL035c*, *YMR034c*, *ICY2*, *PRY1*, *YDL124w*
Aft2	Transporters	*SMF3*, *MRS4*, *FTR1*, *COT1*
	Cu chaperon	*ATX1*
	Ferroxidase	*FET3*, *FET5*
	Metalloreductase	*FRE1*
	Cell wall protein	*FIT1*, *FIT3*, *FIT2*
	Fe-S biosynthesis	*ISU1*
	Other	BNA2, *ECM4*, *LAP4*, *TIS11*, *YOL083w*, *YGR146c*, *YHL035c*
Ace1	Cu MTs	*CUP1*, *CRS5*
	Cellular stress response	*SOD1*
Mac1	Cu transporters	*CTR1*, *CTR3*
	Metalloreductases	*FRE1*, *FRE7*
	Other	YFR055w, *YJL217w*, *YLR213c*
Cuf1	Cu transporters	*ctr4*+, *ctr5*+, *ctr6*+
	Fe transport	*fip*+ *
	Metalloreductase	*frp1*+ *
	Multicopper oxidase	*fio1*+ *
GRISEA	Cu transport	*PaCTR3*
	Cellular stress response	*PaSOD2*
Zap1	Zn transporters	*ZRT1*, *ZRT2*, *ZRT3*, *ZRC1*, *FET4*, *ZRG17*
	Gene regulation	*ZAP1*, *NRG2*
	Phosph/lipid metabolism	*YOL002c*
	Metabolic enzymes	*DPP1*, *ADH4*, *MNT2*, *ADE17*, *TKL2*, *URA10*
	Vacuolar proteases	*PRC1*, *PEP4*
	Other	MCD4, *ZPS1*, *RAD27*, *ZIP1*, *GRE2*, *BAG7*, *FLO1*, *YNL254c*, *YOL131w*, *YGL258w*, *YOR387c*, *YJR061w*, *YMR086w*, *YLL020c*, *GPG1*, *COS1-4,6,8*, *YJL132w*, *ICY2*, *PST1*, *PHM7*, *YBL048w*, *YBL049w*, *YNL234w*, *YDR492w*, *YKL174c*
Mammalian MTF-1	Zn transporters	*ZNT-1*, *ZTL1*
	MTs	*MT-I*, *MT-I*
	Cellular stress response	*AFP*, *LCN1*, *PIGF*, γ-*GCS*
	Other	*AHSG*, *CBG*, *PMP22*, *XIST*, *ACVR2b*
Drosophila MTF-1	MTs	*MTNA*, *MTNB*, *MTNC*, *MTND*

Adapted from Rutherford and Bird, Eukaryot Cell. 2004, modified

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Transcriptome Response of *Drosophila* to the Heavy Metals Cadmium, Copper and Zinc: Similarities and Differences

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Key words: cadmium, zinc, copper, microarray, *Drosophila*, metallothioneins, glutathione-S-transferases, metal transport

Abstract

All organisms have to ensure metal homeostasis and be able to cope with fluctuating amounts of trace elements in the environment. In order to provide insight into how transcriptional mechanisms contribute to maintain heavy metal homeostasis and protect against heavy metal stress, we have determined the transcriptome response in *Drosophila* after short exposures to sublethal doses of Cd^{2+}, Cu^{2+} and Zn^{2+}. Results indicate that the gene family encoding metallothioneins, which are small, cysteine-rich proteins with a high affinity to heavy metals, was activated by all metals, but individual metallothioneins showed metal-specific differences. Two other genes, *CG10505*, homolog of "yeast cadmium factor" (YCF1), and *CG10404*, a gene of unknown function, were also induced by all three metals. Two *Drosophila* Delta class glutathione S-transferases, *GstD2* and *GstD5,* were induced dramatically by Cd^{2+} and Zn^{2+}, suggesting a role in metal detoxification and /or protection against metal-induced oxidative stress. We also studied the transcriptome response to copper depletion by adding a copper chelator to the food. In contrast to the strong induction observed upon heavy metal load, metallothioneins were dramatically down-regulated by copper depletion. This underscores the role of metallothioneins in the sequestration / detoxification of heavy metals. In addition to metal-protective genes, the expression of metal transport systems was affected by the various treatments. For example, zinc transporter homologs were induced by Zn^{2+} and Cd^{2+}, while the copper importer *Ctr1B* was specifically repressed by Cu^{2+} and induced by Cu^{2+} depletion. Taken together, *Drosophila* controls metal homeostasis and detoxification

via expression of metal transport systems, proteins involved in metal sequestration and proteins involved in defense against toxic effects, including oxidative stress. Whereas the transcriptional regulation for most of the differentially expressed genes needs to be elucidated, several of them harbor metal-response elements, indicating the possible role of the metal-responsive transcription factor MTF-1 in their regulation.

Introduction

Every living cell is challenged to keep out non-essential, toxic heavy metals, such as cadmium*, mercury and lead, and strictly maintain physiological levels of essential but potentially toxic metals. A good example of the latter is copper, an essential element for living systems, as it is required for the catalytic activity of a number of important enzymes involved in oxidative stress protection, ATP synthesis, iron transport, wound healing and development (1). However, despite being an essential nutrient, copper is highly toxic when in excess and can catalyze the synthesis of reactive oxygen species.

Zinc is another essential metal and is a catalytic component of over 300 enzymes (2). It is not redox-active under physiological conditions and is considerably less toxic than copper. However, at high concentrations zinc may bind to inappropriate sites in proteins or cofactors and interfere with their functions. Zinc toxicity has been found to be associated with reduced iron absorption (3), impaired immune function (4) and neuronal death (5,6).

Cadmium, a non-essential and toxic metal, does not undergo redox reactions. Nevertheless, it depletes glutathione and protein-associated sulfhydryl groups, thus resulting in enhanced production of reactive oxygen species (7). A number of deleterious effects have been reported for cadmium. It can replace zinc, and can substitute for calcium in bone (8,9). It has also been reported that cadmium induces apoptosis (10), activates the transcription of protooncogenes such as c-jun, c-fos, c-myc, and mdm2, inhibits tumor suppressor genes such as p53 and p27 (11-13), causes single stranded DNA breaks (14), and, as shown recently, inhibits DNA repair (15).

Consequently, biological systems have evolved mechanisms to ensure metal homeostasis and to protect themselves against heavy metals and their deleterious effects. Increased metal export, reduced import and metal ion sequestration are direct and widely

used mechanisms as a first line of defense. In addition, metals can trigger a diversity of responses, such as apoptosis, heat shock response or oxidative stress response, which protect the organism against harmful effects of metals. Znt proteins, transmembrane transporters of CDF (cation diffusion facilitator) family, maintain intracellular zinc concentrations through sequestration and efflux in response to high zinc levels (reviewed in (16,17)). On the other hand, many members of the ZIP (ZRT (zinc regulated transporter) and IRT (iron regulated transporter) like protein) superfamily, which mediate zinc uptake in zinc deficiency, are down-regulated upon zinc load (17). CDF and ZIP family genes have been found in many eukaryotes from yeast to mammals. Likewise, we report here the existence of genes homologous to yeast and mammalian zinc transporters in the *Drosophila* genome, indicating that the mechanisms of zinc homeostasis are conserved.

Copper acquisition occurs through the Ctr1 family of transporters, in yeast and mammals (18-22). Recently, three high-affinity copper importers, *Ctr1A, Ctr1B* and *Ctr1C,* have also been characterized in *Drosophila* (23). Two P-type ATPases, Menkes (MNK) and Wilson disease (WD) proteins, have been shown to be important for cellular copper export in mammals (reviewed in (24)). MNK exports copper from the enterocytes into the portal circulation and thus provides the body with this essential metal. WD is expressed in hepatocytes and in response to copper overload it exports excess copper into the bile. There is one homolog of MNK/WD proteins in *Drosophila* (25).

In addition to transport, sequestration of heavy metal plays a major role in metal homeostasis. Metallothioneins (MTs) are a group of small, cysteine-rich proteins with an outstanding metal binding capacity found in all eukaryotes and a number of prokaryotes. Transcription of many MT genes is strongly induced by zinc, copper and cadmium. In the mouse, a double knockout of MT-1 and MT-2 results in higher sensitivity to cadmium and to elevated zinc concentrations (26,27). MT gene expression is dependent on the metal transcription factor-1 (MTF-1), a zinc-finger factor that binds to the metal response elements (MREs) present in the promoters of MTs and some other genes. Four metallothioneins (*MtnA-MtnD*) have been described in *Drosophila* (28-30). The KO of dMTF-1 is extremely sensitive to cadmium, copper or zinc load (28), and the

metallothionein family KO in Drosophila is particularly sensitive to cadmium and copper (Egli et al, submitted).

Even though great progress in the biology of transition metals has been made, a detailed analysis and a comparison of the transcriptional response to cadmium, copper and zinc has not been reported in a higher organism. As misregulation of copper and zinc-homeostasis can lead to disease, and cadmium poses a significant health risk to animals and humans, there is considerable interest in understanding how the organism coordinates the defense response at the transcriptional level. *Drosophila* is a convenient system for such a study since many aspects of metal homeostasis are conserved in this model organism.

Here we have undertaken a study of the *Drosophila* transcriptome response to sublethal doses of cadmium, zinc and copper, as well as to copper deficiency. The key mechanisms known to be part of the defense against metal toxicity, such as metal transport, metal sequestration and oxidative stress response, were found induced in *Drosophila* larvae after six hours of metal exposure. All four *Drosophila* metallothioneins were significantly induced by all three metals tested. The responses to different heavy metals, especially to zinc and cadmium, have common features, but also interesting differences reflecting the distinct biology of each.

* The designations Cd or cadmium, Cu or copper and Zn or zinc are conventionally used here to denote Cd^{2+}, Cu^{2+} and Zn^{2+} and ions, respectively.

Materials and Methods

Fly food and RNA extraction

Animals were raised on standard cornmeal molasses-based food. In the 3rd larval instar (fourth day of the development) the animals were transferred from normal to supplemented food containing either 0.05 mM $CdCl_2$, 0.5 mM $CuSO_4$, and 5 mM $ZnCl_2$. After six hours of feeding on the supplemented food, RNA was extracted. For the case of the treatment with the copper chelator BCS (bathocuproine disulfonic acid), the animals were kept continuously in the chelator-containing food, since copper stores of a normally fed animal cannot be expected to be exhausted within a few hours. In BCS larval development is delayed, so the RNA was extracted on the fifth day after egg laying. To control for the handling of the larvae during transfer to supplemented food, the normal food controls and the larvae grown in BCS were also removed at 3rd instar and transferred for the last six hours to normal or BCS containing food, respectively. Total RNA was extracted using TRIzol reagent (Life Technologies).

S1 nuclease protection assay

Nuclease S1 protection assay with 150 or 200μg was performed as described (36). The dried gels were exposed to storage phosphor screens and analyzed using a PhosphorImager (Molecular Dynamics).

Microarray

Microarray experiments were done in triplicates using a pool of RNA from at least 30 animals for each assay. cDNA was synthesized from larval total RNA with SuperScript reverse transcriptase (Invitrogen/Life Techn. cDNA Synthesis Kit) using T7-(T)24 primer 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTT TTTTTTTTTT-3'. The resulting cDNA was purified with Phase Lock Gels and concentrated by ethanol precipitation. Synthetic double-stranded cDNA was in vitro transcribed into biotin-labeled cRNA with biotinylated 11-CTP and 16-UTP (Ambion MEGAscript T7 kit). Biotin-labeled cRNA was then isolated with an RNeasy Mini Kit (Qiagen). 15 μg adjusted cRNA was taken for fragmentation. 11.5 μg fragmented (50-

200-nucleotide pieces) cRNA was used to probe the *Drosophila* Genome Array (Affymetrix), which contains more than 13,500 mRNA transcripts from known *Drosophila* genes and predicted ORFs. Probe arrays were treated with streptavidin, anti-streptavidin goat antibody, biotinylated goat IgG antibody and stained with streptavidin phycoerythrin. Arrays were scanned twice with a Agilent G2500 Genome Array Scanner.

Software and Statistical Analysis

Raw data processing was performed using the Affymetrix Microarray Suite Ver. 5.0 (MAS5) Software. After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS5 algorithm (37). In order to compare the expression values of the genes from chip to chip, global scaling was performed resulting in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500 as described in the Statistical Algorithms Description Document (Affymetrix, 2002). Quality control measures were considered before performing the statistical analysis. These included adequate scaling factors (between 10 and 17 for all samples) and appropriate total number of "Present Calls" per chip (26-30%) calculated by application of a signed-rank call algorithm (38). Furthermore, the optimal 3′/5′ hybridization ratios (around 1) for the housekeeping genes (GAPDH, actin) as well as for the spike controls (BIOB, BIOC, CREX, BIODN), added as hybridization controls into the hybridization cocktail were taken into consideration. After filtering of genes with unreliable expression, using the Cross-Gene Error Model implemented in the Gene Spring software 5.1. (Silicongenetics, 2003) unequal variance t-test was applied to detect significantly differentially expressed genes. In general, a significance level of 0.05 was chosen. Furthermore, the signal-rank call algorithm from the MAS5 software (38) was applied as an additional filter. Within one comparison of two conditions, each gene was taken into account for further analysis if the algorithm attributed "Present Calls" to at least 50% of the values.

Results

Drosophila larvae have a remarkable ability to cope with a wide range of heavy metal concentrations in the ingested food. Wild type flies survive up to 3-4 mM copper, 300 μM cadmium and 15 mM zinc, concentrations which are much higher than what is found under standard conditions. Elevated metal concentrations in the food also result in a dramatic increase of copper, cadmium or zinc content of the body (fig.1, see also (Ballan-Dufrancais 2002)). When the copper concentration in the food rises from 5 μM Cu in normal food to 500 μM copper, total body copper increases from 5ng/mg to 80ng/mg. Total body zinc-content rises about 7 fold from normal food (200 μM zinc) to 4 mM zinc. Total body copper and zinc therefore do not increase proportionally to the metal concentration in the food, suggesting the existence of homeostatic mechanisms regulating metal uptake and efflux. The ability of *Drosophila* to prevent the toxic effects of excessive metal while ensuring the functionality of metal-dependent processes requires a number of responses. To elucidate the cellular response to heavy metals, we conducted microarray experiments with RNA from *Drosophila* larvae kept on food containing 50 μM $CdCl_2$, 500 μM $CuSO_4$ or 5 mM $ZnCl_2$ for six hours. To gain more insights into metal acquisition and homeostasis, we also performed microarrays with RNA from larvae kept on food containing a copper chelator, bathocuproine disulfonic acid (BCS). To assess the transcriptome response of the whole organism we have chosen to use RNA from whole larvae. One caveat of this approach is that any differential expression of genes with a restricted tissue specificity could be underestimated.

Genes differentially expressed upon cadmium treatment

After applying statistical filters (see Materials and Methods) and using a 2-fold difference as a threshold change we observed 10 genes up-regulated and 3 down-regulated in response to 50 μM Cd (Table Ia). Metallothioneins were highly induced, thus confirming their role in cadmium detoxification (39). Two genes of the insect Delta class glutathione S-transferases, *GstD5* and *GstD2*, were also significantly up-regulated (Table Ia). GSTs comprise a large family of detoxification enzymes conserved from yeast to mammals. They have been shown to be induced by a wide range of chemical agents (40-42), reactive oxygen species, heat shock, fungal and bacterial infections (43), and also by

heavy metals. In human cells cadmium, arsenic and chromium induce GST Ya, and some heavy metals, including cadmium and zinc, were shown to markedly induce tau class GSTs in rice (44,45). An ATP-binding cassette (ABC) transporter, *CG10505*, was induced 3.3 fold. This transporter shares 19% identity with S. cerevisiae YCF1 (yeast cadmium factor) protein, which confers cadmium resistance in yeast (46) and 25% identity with human multidrug resistance associated protein (MRP), which confers resistance to various cytotoxic drugs by exporting them from the cell. Cadmium also elevated the expression of the *Hsp22* gene in *Drosophila* larvae. This is consistent with the observed heat shock response induced by cadmium in yeast (35,47) mice (48), and various human cell lines (49,50).

If the criteria for differential expression are lowered to factor 1.5, 27 additional genes appear in the list (Table 1). Interestingly, two of them code for zinc exporter-related proteins (*CG3994* and *CG17723*).

Genes differentially expressed upon copper load and scarcity

The larvae exposed to 500 μM Cu induced the transcription of six genes more than two fold. Another six genes were down-regulated in this condition (Table II). As expected, metallothioneins were up-regulated, implying a role in protection against toxic effects of copper. Curiously, the other three transcripts up-regulated by high copper levels all code for proteins involved in immune defense against microbial infection. Only five genes were added to the list when the 1.5 fold induction criterion was used. The high-affinity copper importer, *Ctr1B* was strongly down-regulated in response to copper treatment (Table IIb). Two other copper-importers, *Ctr1A* and *Ctr1C*, showed no changes in their mRNA levels.

The transcriptome response to the five-day exposure to 500 μM BCS was more pronounced, with 138 genes being differentially expressed (Table III and suppl. data). Under conditions of copper deprivation, transcripts of the copper importer *Ctr1B* were increased more than 4-fold (Table IIIa). Also, five *cytochrome P450* genes were induced, while another four were down-regulated between 1.6 to 5.3 fold by BCS treatment. P450 enzymes have a wide spectrum of enzymatic activities, such as synthesis and degradation of ecdysteroids or metabolism of foreign chemicals. P450 enzymes metabolize many

compounds such that they can serve as direct GST substrates (32). Consistent with reduced copper levels affecting copper containing proteins, scarcity of copper might result in oxidative stress due to compromised Cu-Zn SOD (superoxide dismutase) activity. In line with this, we observed an about 7 fold up-regulation in *GstD5* expression. Furthermore, metallothioneins were significantly down-regulated by Cu depletion (Table IIIb). In this context it is worth mentioning that others have observed that under conditions of extreme zinc deprivation, in a mouse fibroblast cell line, metallothionein mRNAs are highly expressed. It is suggested that the elevated MT protein participates in a zinc-scavenging system which serves as a reservoir of zinc during times of zinc scarcity (51). For copper, however, our data do not support a role for metallothioneins as chaperones, as the levels of metallothionein expression are decreased upon copper deprivation. Rather our data only support MT scavenger function during copper excess. The expression of known *Drosophila* copper chaperones, *CCS, CG8885* and *CG9065* were not changed by high or low copper conditions.

Genes differentially expressed upon zinc treatment

In contrast to the limited number of genes responding to cadmium and copper, the larval transcriptome response to zinc was more extensive. Although it is difficult to directly compare the concentrations of the different metals applied, the extent of up-regulation of the very same genes (i.e. *MtnA, MtnB* or *GstD5,* see Tables I, II, IV) in all cases suggests that the conditions used result in approximately the same toxicity. When setting the significance limit at a 2-fold difference in expression, the transcripts of 110 genes were up-regulated in response to 5 mM Zn. Again, metallothioneins (*MtnB* and *MtnC*) were residing at the top of the gene list (Table IVa). Two Zn transporter genes, *CG17723* and *CG3994* were also induced, though less dramatically. *CG3994* emerged when less stringent statistical criteria were taken (p-value 0.06). In addition to the zinc transporters, the same glutathione S-transferases up-regulated in cadmium, namely *GstD5* and *GstD2*, were induced 12.5 and 3.3 fold by zinc, respectively. Three further GSTs were induced to a lesser extent. Another cadmium inducible gene, the YCF1 homolog ABC transporter (*CG10505*) was more than 5 fold up-regulated by zinc, suggesting similar mechanisms of

metal defense in response to cadmium and to elevated zinc levels. Furthermore, gamma-glutamyl transferase, a gene involved in glutathione metabolism, was induced 2.8 fold.

A number of genes with possible cell-protective function were up-regulated by elevated zinc levels, for example lysozyme (*LysX,* 2.2 fold induction) and the potential apoptosis inhibitor (*CG17019*, 6 fold induction) (Table IVa, suppl. data). However, the up-regulation of several members of the same gene family, or clusters of genes involved in the same pathway, may be a more reliable indication for their involvement in heavy metal stress. In this respect, 8 *cytochrome P450* genes were up-regulated more than 1.5 fold by zinc; one of which was also induced by copper (suppl. data, Table IIa). Another group of up-regulated genes was the ubiquitin conjugating enzymes. Three of these were induced between 1.6 to 1.8 fold. Interestingly, the genes encoding the iron storage proteins ferritin 1 heavy chain homologue (Fer1HCH) and ferritin 2 light chain homologue (Fer2LCH) were up-regulated 2 and 3 fold, respectively (see also below). These ferritins were also induced between 1.7 to 2 fold in response to elevated Cd levels, but statistically less significant (*Fer1HCH*, p-value 0.15; *Fer2LCH,* p-value 0.22).

Common genes in metal response

Next, we combined the genes induced at least 1.5 fold in all tested metal conditions (Fig. 2a). All four *Drosophila metallothioneins* were induced by cadmium, copper and zinc. In addition, *CG10404 (CG30152),* a gene of unknown function was also induced by all three metals. *CG10505*, the ABC transporter that is related to yeast cadmium factor was found to be induced only by zinc and cadmium, but transcript mapping experiments show induction also in copper (Fig.3c). Ten genes were in common between cadmium and zinc but not with copper. When we compared the data in order to see what genes were down-regulated greater than or equal to 1.5 fold by cadmium, copper and zinc load, only *CG10073*, a gene of unknown function was down-regulated in all metals (Fig 2b). Curiously, the same gene was also found to be 77 fold down-regulated in copper depletion. However, 15 genes were down-regulated by both zinc and copper treatments.

Microarray data validation

We also tested the transcript level of several differentially expressed genes in an S1 nuclease protection assay. As expected, *MtnA* gene expression was elevated by copper, and it was gradually down-regulated by increasing concentrations of BCS (Fig. 3a). The zinc transporter *CG3994* was more than three fold induced by both cadmium and zinc (Fig. 3b), and the expression was not changed by copper load. The ABC transporter *CG10505* was induced not only by cadmium and zinc but also by copper treatment (Fig. 3c). Similar induction was observed with *CG30152 (CG10404)* (Fig. 3d). Ferritin transcripts were significantly up-regulated in response to high cadmium and zinc concentrations (Fig. 3e). We checked also the expression of *GstD2* and *GstD5* genes by semi-quantitative RT-PCR. The larvae fed with metals had clearly higher expression of both *Gst* genes (data not shown). The results described above are in agreement with the microarray data.

Metal response elements in the promoters

Metal response elements (MREs) are short DNA sequence motifs in the enhancer/promoter region of metal-regulated genes, notably metallothioneins or metal exporters (52,53). MREs are recognized by the zinc finger transcription factor *MTF-1*. In *Drosophila, MTF-1* is essential for both basal and heavy metal induced expression of all four MT genes (39). However, similar to the situation in mammals (54), the expression of *dMTF-1* itself was not changed in response to high metal concentrations.

We checked the promoter regions of differentially expressed genes as determined by our microarray experiments for the presence of MREs. Several of the metal induced genes indeed contain MREs (Tables I, II, IV) suggesting that *dMTF-1* may be involved in their activation, as was recently shown to be the case with the copper transporter *Ctr1B* (55).

Discussion

For our microarray experiments we took RNA from Drosophila larvae treated with excess copper, zinc or cadmium, or with the copper chelator BCS. An advantage of the larval stage is that animals continuously take up nutrients. The purpose of choosing relatively mild sublethal concentrations and a six hours time point was to identify primary metal response genes, rather than genes involved in a cell damage response or secondary adaptation.

In cadmium, copper, and zinc treatments, the most dramatically up-regulated transcripts were those of the metallothionein genes. This universal induction is in line with a protective role of metallothioneins against metal toxicity. There were however differences in the strength of the response of individual MT genes. Remarkably, *MtnA* induction was always between 3 and 4 fold in each of the metals (Tables Ia, IIa, IVa), whereas *MtnB and MtnC* were more strongly induced by zinc and cadmium (9-17 fold) than by copper (8 and 6 fold). This might suggest specific roles for metallothioneins in scavenging different metals. In absolute terms, expression of *MtnA* was higher than that of other metallothioneins, with an impressive basal level and a relatively modest induction, suggesting *MtnA* to be the major metal scavenger during the larval stage of *Drosophila* life. The *MtnD* gene was annotated only recently and not represented in the *Drosophila* genome arrays; however, its metal inducibility has been independently confirmed ((28) and data not shown).

A second, intriguing, group of up-regulated genes encode a subset of glutathione S-transferases (GSTs), a major family of detoxification enzymes. GSTs catalyze the conjugation of glutathione to a variety of harmful compounds. These GSH conjugates are further metabolized and are, ultimately, sequestered in vacuoles and/or excreted. The mechanism of these processes varies with different compounds and in different species. In *S. cerevisiae*, for example, cadmium ions are conjugated to GSH and the $Cd{\cdot}GS_2$ conjugate is transported into the vacuole by yeast cadmium factor (YCF1), an ATP-binding cassette transporter (56). *Drosophila* GSTs are grouped into six classes; Delta, Sigma, Theta, Zeta, Omega and Epsilon (40,57). In total there are more than 40 GST genes in *Drosophila*. However, there is little information on specific functions attributable to a single family or family member. Here we provide evidence for such a

specific function in that two *Drosophila* Delta class glutathione transferases, *GstD2* and *GstD5,* were dramatically induced by zinc and cadmium. Interestingly, although there are at least ten D class GSTs in *Drosophila* (58), only two of them changed in both high zinc and cadmium, suggesting specific roles in heavy metal stress response. Three GSTs, *CG17524, CG6776*, and *CG1681* with homology to epsilon, omega, and theta class GSTs, respectively, were up-regulated by zinc. It is plausible that the protein products of these genes catalyze the conjugation of Cd and/or Zn to GSH, which eventually will be removed from the cytoplasm. Alternatively, or in addition to conjugating the metal, they might conjugate the ROS, byproducts of the metals, to GSH. Interestingly, a specific ATP-binding cassette transporter, a homolog of yeast cadmium factor, is up-regulated more than 5 fold by zinc and more than 3 fold by cadmium and copper. It is tempting to speculate that this ABC transporter contributes, in analogy to the situation in yeast, to $Cd{\bullet}GS_2$ export in *Drosophila*. Another enzyme, gamma-glutamyl transferase (GGT), an integral part of the gamma-glutamyl cycle involving the degradation and neo-synthesis of GSH, is also involved in GSH conjugate metabolism and excretion (31). A *Drosophila* GGT was induced by zinc, suggesting a similar processing of glutathione conjugates as is found in other organisms. Taken together, it seems likely that GSH-mediated detoxification plays a major role in the response of *Drosophila* to zinc and cadmium stress.

Interestingly, in yeast, cadmium exposure can globally modify the proteome by reducing the production of abundant sulfur-rich proteins, thus saving sulfur for elevated GSH production (59). When we checked the cysteine and methionine contents of the differentially expressed proteins we could not detect such a change. However, such a regulation may become obvious only after long-term exposure to metals; moreover, some of the rearrangements of the proteome could take place post-translationally.

Another essential system for metal ion homeostasis is metal export and import. Although transcriptional change is only one of several regulatory processes involved, we could follow logical changes in characterized, as well as putative, *Drosophila* metal transporters. Apparently, to provide adequate intracellular copper concentrations, the high affinity copper importer *Ctr1B* is strongly induced by copper depletion; conversely, it is down-regulated about four-fold at high Cu concentration in order to reduce copper

uptake. Two zinc transporter genes (*CG3994* and *CG17723*), both encoding putative ZnT family members involved in zinc efflux, were up-regulated by zinc, as well as by cadmium treatments. The up-regulation of these transporters by both metals points to their role not only in zinc ion homeostasis but also in defending against cadmium toxicity.

Interestingly, several ubiquitin conjugating enzymes (*Ubc*) were induced by zinc. This suggests the involvement of a post-translational mechanism in handling zinc stress. In this context it is worth mentioning the specific degradation of the zinc importer Zrt1p in yeast upon zinc load (60). The up-regulation of *Ubc* might help to degrade misfolded proteins which are probably formed upon metal load.

Of particular interest is the induction of ferritin 1 heavy chain homologue *(Fer1HCH)* and ferritin 2 light chain homologue *(Fer2LCH*) genes (Table 4a, Fig. 2b, suppl. data). Ferritin is well recognized as a protein which detoxifies, stores and transports iron. It was also shown to bind other metal ions such as Cu, Zn, Cd, Be, Pb and Al and has been suggested to function as a general metal detoxicant in mammals (61). Moreover, it may function as a zinc and copper ion donor (61,62). Here we report an up-regulation of *Drosophila ferritins* by cadmium and zinc, which suggests a detoxifying role for the *Drosophila* protein. Interestingly, both *Fer1HCH* and *Fer2LCH* contain multiple metal-response elements (MREs) in their upstream regions, indicating a possible role of *dMTF-1* in their regulation.

Evidently, the larval transcriptome response to zinc and cadmium shares several elements. The chemical properties of these metals are similar. Cadmium can replace zinc in the tissues and in enzyme binding sites, although this interaction is, apparently, not optimal, and is possibly deleterious. Hence it is not surprising that the regulatory mechanisms handling excess zinc are also functional in cadmium stress.

Only a few genes were up-regulated in response to 500 μM Cu. Copper can induce the production of free radicals, but at the concentration used, there was no up-regulation of radical scavengers, other than metallothioneins, such as superoxide dismutase (SOD) or catalase. However, there was a robust induction of three genes encoding antimicrobial peptides; attacins, and gram-negative antibacterial peptide. Interestingly, also in zinc and cadmium treatments several defence genes appeared, such

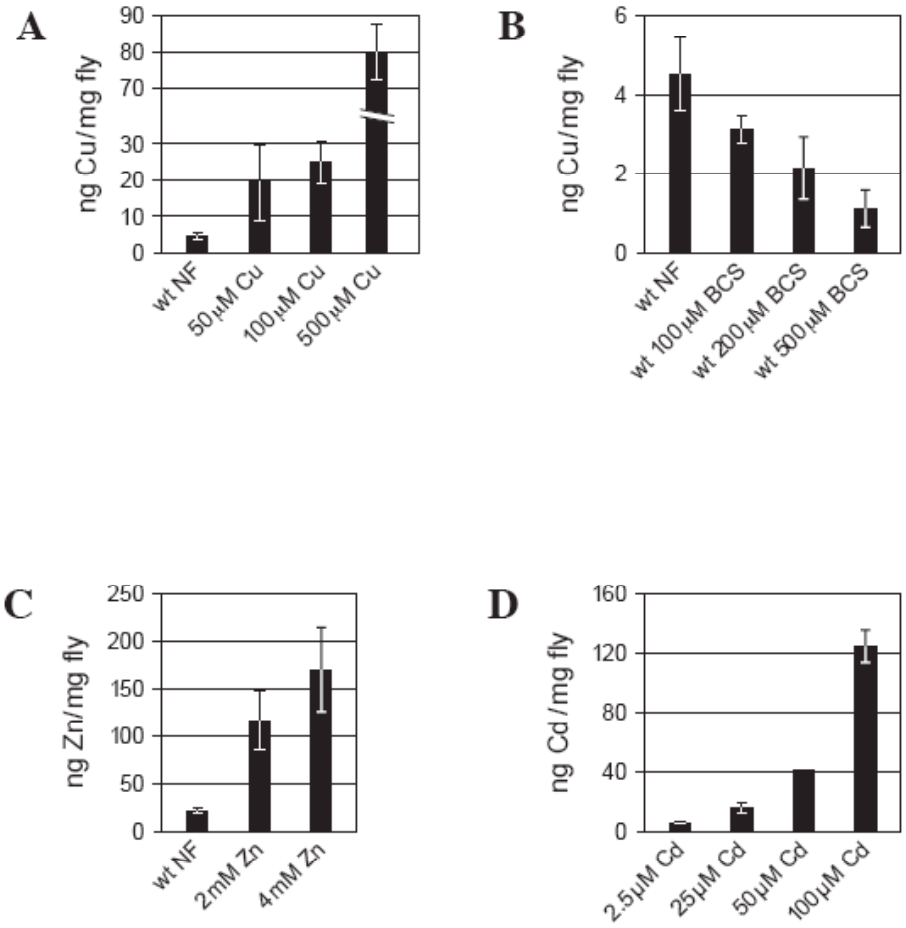
as ones encoding peptidoglycan recognition proteins CG14745 and CG11709 and drongo (see supplementarty data). This could represent an ectopic induction, or perhaps metal stress could make the larvae more vulnerable to microbial attack which results in such a countermeasure. However, when we challenged the null mutants of *Drosophila* metal transcription factor MTF-1 with Gramm positive, Gramm negative bacteria or fungi, no significant changes in viability were observed (data not shown).

By contrast to the low number of genes affected by Cu load, many genes changed their expression when Cu was depleted by the copper chelator BCS. Unlike the short-term metal treatments, the animals were allowed to develop in the chelator-containing food from the egg. The deleterious effect of copper depletion had already manifested itself at the phenotypic level. Larval development was delayed for at least one day, from four to five days, in 500 μM BCS. The changes in the gene expression can be expected to reprogram the cell to maximize copper acquisition, to maintain the scarce metal as well as to counteract any disorders resulting from the altered cellular metal metabolism and reduced activity of copper enzymes. The up-regulation of *Ctr1B*, the high-affinity copper transporter, is a particularly nice example of such an adaptation, whereby this gene was down-regulated under conditions of excess copper but induced under copper starvation conditions. Zinc and cadmium, however, did not affect *Ctr1B* expression. On the other hand the zinc transporter *CG3994* was specifically induced by zinc and cadmium and remained unchanged upon copper load. The next challenge will be to elucidate the mechanisms of this metal specificity.

Acknowledgements

We thank the Functional Genomics Center Zurich (FGCZ) for advice and for financial support. We are grateful to Dr. Ulrich Wagner for assistance in microarray data analysis, Andrea Patrignani and Bruno Schmid for technical support, Fritz Ochsenbein for preparation of figures, Dr. George Hausmann and Dr. Michael Fetchko for critical reading of the manuscript. This work was supported by Kanton Zürich and by the Swiss National Science Foundation.

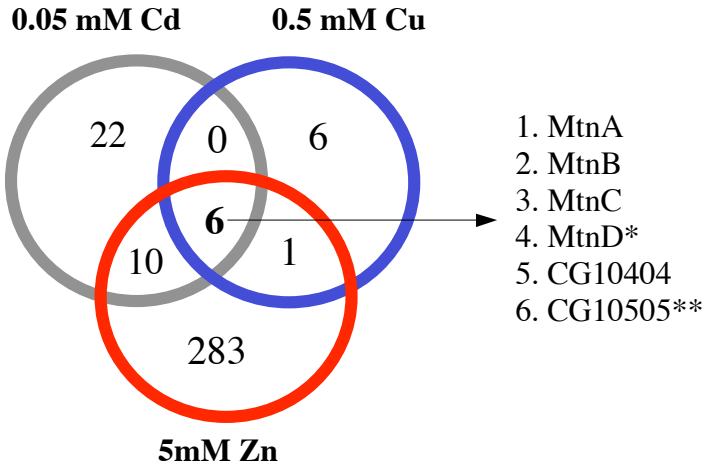
Figure 1



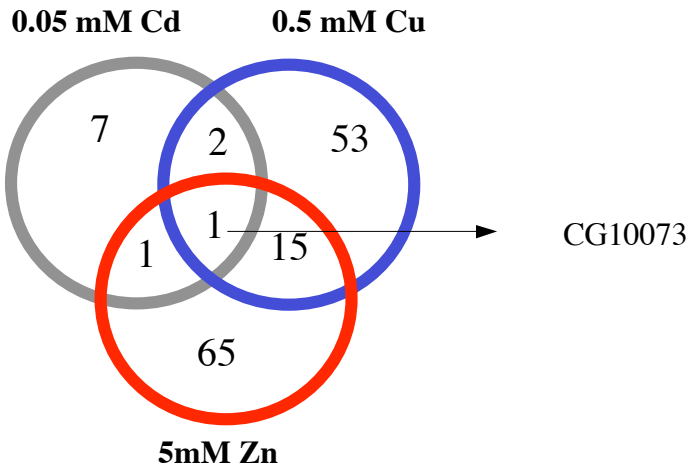
Metal amounts (nanogram of metal per milligram of fly) in the flies grown on normal food or food supplemented with indicated concentrations of copper (**A**), copper chelator BCS (**B**), zinc (**C**) and cadmium (**D**).

Figure 2

A.

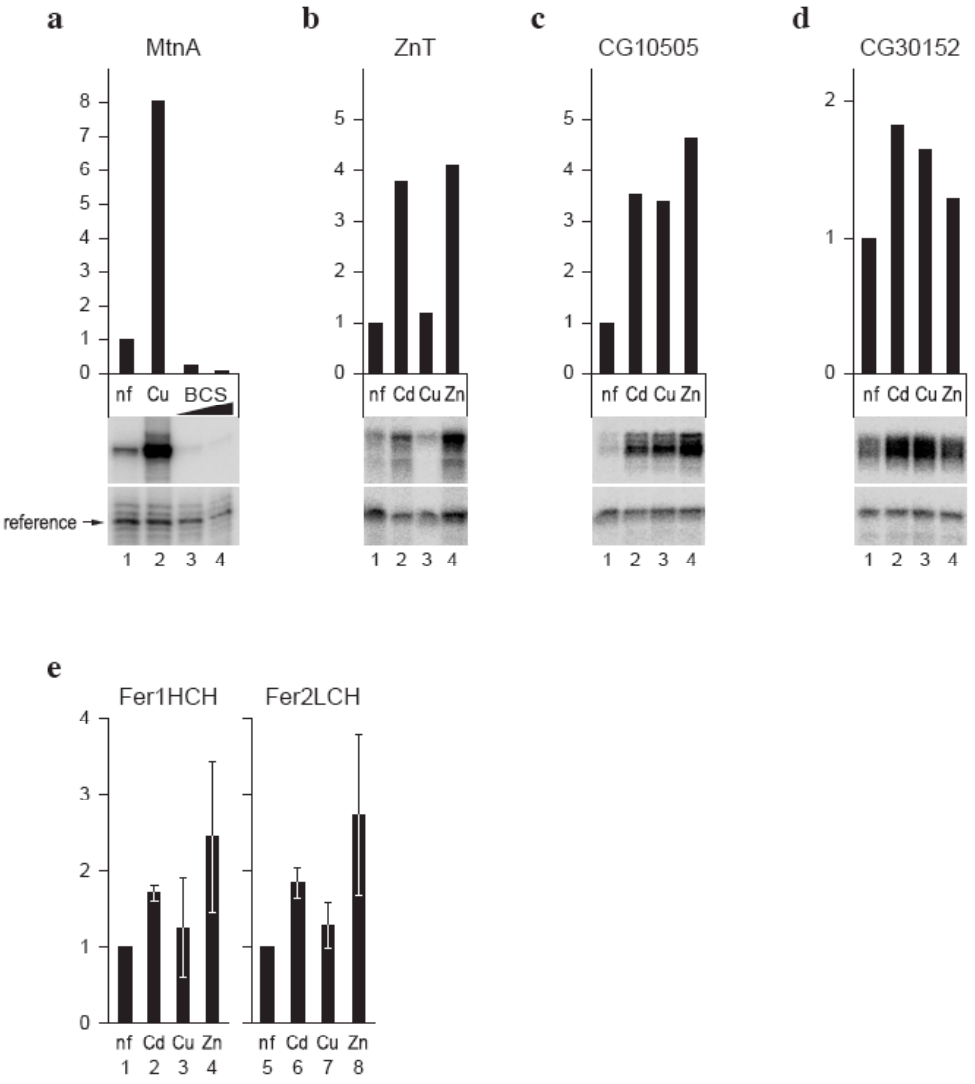


B.



Venn diagrams of differentially expressed genes in 50 uM Cd, 500 uM Cu and 5 mM Zn
(A) Genes up-regulated more than 1.5-fold, p-value ≤ 0.05
* The *MtnD* gene was not represented in the *Drosophila* genome arrays; it has been found to be induced by metals in an S1 nuclease protection assay (data not shown).
** *CG10505* was strongly induced both by cadmium and zinc load in the microarray, and less than 1.5-fold by copper. S1 nuclease protection assay, however, shows more than 3 fold up-regulation in copper load.
(**B**) Genes down-regulated more than 1.5-fold, p-value ≤ 0.05

Figure 3



Transcripts of several genes were determined by S1 nuclease protection assay. nf = normal food, Cd = 50 uM $CdCl_2$, Cu = 500 uM $CuSO_4$, Zn = 5 mM $ZnCl_2$. References are actin5 (a) or tubulin85 (b, c). Bar diagrams represent quantitation of normalized data. (**a**) *MtnA* is dramatically up-regulated by copper (lane 1 versus 2), and gradually down-regulated by copper depletion resulting from increasing concentrations of copper chelator, BCS. Lane 3; 0.3 mM BCS, lane 4; 0.5 mM BCS (**b**) Zn transporter *CG3994* transcript levels are 3.5-fold induced by Cd and Zn (compare lane 1 to lanes 2 and 4), but are not changed by Cu. (**c**) ABC transporter *CG10505* mRNA is up-regulated by all three metals (compare lane 1 to 2, 3 and 4). (**d**) *CG30152* is moderately induced upon metal load (**e**) *Fer1HCH* and *Fer2LCH* are significantly induced by both Cd and Zn (compare lane 1 and 5 to 2, 4 and 6, 8)

Table I

a. Genes up-regulated more than 1.5 fold in 50 μM cadmium, p-value ≤ 0.05

Gene	fold up-regulation	MREs within 1 kb from transcription (or translation) start
MtnB (Metallothionein B)	11.5	4
MtnC (Metallothionein C)	13.2	5
GstD5 (Glutathione-S transferase D5)	10.1	-
Hsp22 (Heat shock protein 22)	3.4	2
GstD2 (Glutathione-S transferase D2)	3.4	-
CG10505 (ATP-binding cassette transporter)	3.3	1
MtnA (Metallothionein A)	2.7	2
CG10404	2.2	-
Dgp-1	2.1	-
CG8665	2.0	-
Lnk	1.9	-
CG4439	1.9	3
CG18419	1.9	-
CG7763	1.9	2
CG17723 (zinc transporter-like)	1.8	-
CG17487	1.8	4
BetaTub85D	1.8	-
CG3994 (zinc transporter-like)	1.7	2
CG15101	1.7	
CG13771 (acyl-CoA thioesterase)	1.7	2
Dnz1 (DNZDHHC/NEW1 zinc finger protein 11)	1.7	1
CG6778 (glycine-tRNA ligase)	1.7	-
CG17567	1.6	-
CG9764 (yurt)	1.6	-
CG7702 (connectin-like)	1.6	4
CG3522	1.6	1
CG11139	1.6	1
crq (croquemort)	1.6	1
CG4931 (Sra-1)	1.6	4
CG11709 (peptidoglycan recognition protein SA)	1.6	-
CG3332	1.6	-
CG6263 (ATPase)	1.5	2
CG7029	1.5	1
CG5379	1.5	-
Dead-box-1 (ATP dependent helicase)	1.5	2
CG5451	1.5	1
CG13062	1.5	-

b. Genes down-regulated more than 2 fold in 50 μM cadmium, p-value ≤ 0.05

Gene	fold down-regulation	MREs within 1 kb from transcription (or translation) start
CG3344	3.9	-
CG18493	2.1	-
CG10444 (sodium-dependent multivitamin transporter)	2.0	1

Table II

a. Genes up-regulated more than 1.5 fold in 500 μM copper, p-value ≤ 0.05

Gene	fold up-regulation	MREs within 1 kb from transcription (or translation) start
MtnB (Metallothionein B)	8.0	4
CG13422 (Gram-negative antibacterial peptide)	6.0	-
MtnC (Metallothionein C)	6.0	5
MtnA (Metallothionein A)	4.0	2
Attacin-A	3.3	1
Attacin-B	2.8	-
fizzy	1.8	1
CG18550 (yellow-f)	1.8	1
CG1967	1.7	2
CG10404	1.7	-
Cyp12a5 (Cytochrome P450)	1.6	-

b. Genes down-regulated more than 2 fold in 500 μM copper, p-value ≤ 0.05

Gene	fold down-regulation	MREs within 1 kb from transcription (or translation) start
Ctr1B (Copper transporter 1B)	3.9	4
usp (ultraspiracle)	3.1	1
CG10073 (Zn-dependent exopeptidase)	2.4	-
CG10635 (co-chaperonin)	2.1	-
CG7678 (hydrogen-exporting ATPase)	2.1	1
viking (type IV collagen)	2.1	-

Table III
a. Genes up-regulated more than 3 fold in 500 μM BCS, p-value ≤ 0.05

Gene	fold up-regulation	MREs within 1 kb from transcription (or translation) start
CG5550 (Fibrinogen-like)	6.9	-
GstD5 (Glutathione-S transferase D5)	6.8	-
CG4835 (peritrophin/chitinase)	6.4	-
CG11893	6.3	2
CG14120	5.6	1
Cyp28a5 (Cytochrome P450)	5.4	-
CG5999 (glucuronosyltransferase)	4.8	1
CG3819	4.7	-
CG6830	4.5	1
Cyp6a23 (Cytochrome P450)	4.4	-
Ctr1B (Copper transporter 1B)	4.3	4
CG6484 (glucose transporter)	4.3	-
CG5724 (glucuronosyltransferase)	4.0	-
CG7298 (peritrophin-like)	3.8	-
CG13482	3.8	-
alphaTrypsin	3.7	-
CG14820 (metallocarboxypeptidase)	3.5	-
Ugt36Bc (glucuronosyltransferase)	3.5	-
Damm (Death Associated Molecule related to Mch2)	3.4	-
CG5845	3.4	-
Cyp9b2 (Cytochrome P450)	3.3	-
CG15255 (metalloendopeptidase)	3.3	-
CG16965	3.3	-
CG4355 (protein tyrosine phosphatase-like)	3.3	-
CG13805	3.2	1
pst (pastrel)	3.2	1
CG11796 (4-hydroxyphenylpyruvate dioxygenase)	3.2	-
CG10912	3.2	-
CG14872	3.0	-

b. Genes down-regulated more than 3 fold in 500μM BCS, p-value ≤ 0.05*

Gene	fold down-regulation	MREs within 1 kb from transcription (or translation) start
CG10073 (Zn-dependent exopeptidase)	77	-
MtnC (Metallothionein C)	28.6	5
MtnA (Metallothionein A)	26.3	2
CG6283 (triacylglycerol lipase)	13.5	-
CG11300	10	1
CG18606	9.0	-
CG8160	7.5	-
CG17191 (triacylglycerol lipase)	6.8	-
CG10476	5.3	2
Cyp6g1 (Cytochrome P450)	4.3	1
Hsp23 (Heat shock protein 23)	4.2	1
CG7658 (cuticle protein-like)	3.4	-
CG4840	3.4	2

*MtnB is 6.4 fold downregulated, p-value = 0.13

Table IV

a. Genes up-regulated more than 3 fold in 5mM zinc, p-value ≤ 0.05

Gene	fold up-regulation	MREs within 1 kb from transcription (or translation) start
MtnC (Metallothionein C)	16.6	5
GstD5 (Glutathione-S transferase D5)	12.5	-
MtnB (Metallothionein B)	8.7	4
CG5550	7.3	-
CG17019 (apoptosis inhibitor)	6.1	-
CG10505 (ATP-binding cassette (ABC) transporter)	5.4	1
CG13482	5.0	-
Ref(2)P (refractory to sigma P)	4.1	-
CG13796 (glycine transporter)	4.0	-
Cyp6a14 (Cytochrome P450)	4.0	2
CG6484 (glucose transporter)	4.0	-
CG2081	3.9	1
CG10116 (lipoprotein lipase)	3.7	-
CG18240	3.7	-
vrille	3.4	1
CG5999 (glucuronosyltransferase)	3.4	1
CG2064	3.3	1
Esterase P	3.3	-
GstD2 (Glutathione-S transferase D2)	3.3	-
CG11065 (AMP deaminase)	3.2	1
CG14255	3.2	1
CG15309	3.1	-
Damm (Death Associated Molecule related to Mch2)	3.1	2
Fer2LCH (Ferritin 2 light chain homologue)	3.0	4
Odc1 (Ornithine decarboxylase 1)	3.0	1
MtnA (Metallothionein A)	3.0	2
CG7170 (chymotrypsin)	3.0	-
CG17524 (glutathione transferase)	3.0	1

b. Genes down-regulated more than 2 fold in 5mM zinc, p-value ≤ 0.05

Gene	fold down-regulation	MREs within 1 kb from transcription (or translation) start
CG11350	3.6	2
CG5240	3.3	-

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Supplementary data

Normal food vs. 00.5 mM Cd, genes upregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold upregulation	gene
1	150257_at	13.21	FB:FBgn0038790 /sym=CG5097 /name= /prod=metallothionein /func=ligand binding or carrier
2	143277_at	11.47	FB:FBgn0002869 /sym=MtnB /name=Metallothionein B /prod=metallothionein B /func=Cu/Cd binding
3	149759_at	10.09	FB:FBgn0038024 /sym=CG12242 /name= /prod= /func=
4	142836_at	3.374	FB:FBgn0001223 /sym=Hsp22 /name=Heat shock protein 22 /prod=heat shock protein 22 kD /func=heat shock protein
5	149756_at	3.369	FB:FBgn0038021 /sym=CG4181 /name= /prod= /func=
6	147616_at	3.292	FB:FBgn0034612 /sym=CG10505 /name= /prod=ATP-binding cassette transporter /func=ion channel
7	143276_at	2.677	FB:FBgn0002868 /sym=MtnA /name=Metallothionein A /prod=metallothionein A /func=Cu/Cd binding
8	147569_at	2.18	FB:FBgn0034543 /sym=CG10404 /name= /prod= /func=
9	144194_at	2.146	FB:FBgn0027836 /sym=Dgp-1 /name= /prod= /func=translation factor
10	146585_at	2.062	FB:FBgn0032945 /sym=CG8665 /name= /prod= /func=enzyme
11	153697_at	1.993	FB:FBgn0028717 /sym=Lnk /name= /prod= /func=cell cycle regulator
12	152130_at	1.937	FB:FBgn0034132 /sym=CG4439 /name= /prod=cytosol aminopeptidase /func=peptidase
13	153232_at	1.886	FB:FBgn0032121 /sym=CG18419 /name= /prod=P-type ATPase /func=transporter
14	147022_at	1.885	FB:FBgn0033634 /sym=CG7763 /name= /prod=C-type lectin-like /func=ligand binding or carrier
15	154124_at	1.838	FB:FBgn0035432 /sym=CG17723 /name= /prod=zinc transporter-like /func=transporter
16	154106_at	1.782	FB:FBgn0039351 /sym=CG17487 /name= /prod= /func=
17	143393_at	1.762	FB:FBgn0003889 /sym=betaTub85D /name=betaTubulin85D /prod=beta-tubulin /func=cytoskeletal structural protein
18	144224_at	1.712	FB:FBgn0028516 /sym=BG:DS07295.1 /name= /prod=zinc transporter-like /func=metal ion transporter
19	141842_at	1.703	BDGP:GH06241.3prime-hit /maps to FB:FBgn0034404 (/sym=CG15101) and FB:FBgn0034403 (/sym=CG18190)
20	145918_at	1.682	FB:FBgn0031844 /sym=CG13771 /name= /prod= /func=
21	153815_at	1.661	FB:FBgn0027453 /sym=Dnz1 /name=DNZDHHC/NEW1 zinc finger protein 11 /prod= /func=transcription factor
22	152230_at	1.653	FB:FBgn0036477 /sym=CG6778 /name= /prod= /func=glycine--tRNA ligase
23	142807_at	1.646	FB:FBgn0040994 /sym=CG17567 /name= /prod= /func=
24	153735_at	1.637	FB:FBgn0038155 /sym=CG9764 /name= /prod= /func=protein phosphatase
25	141382_at	1.626	FB:FBgn0038638 /sym=CG7702 /name= /prod= /func=cell adhesion
26	154214_at	1.608	FB:FBgn0035028 /sym=CG3522 /name= /prod=cholesterol transfer protein-like /func=transporter
27	151810_at	1.58	FB:FBgn0033179 /sym=CG11139 /name= /prod= /func=
28	143858_at	1.57	FB:FBgn0015924 /sym=crq /name=croquemort /prod= /func=macrophage receptor
29	141285_at	1.569	FB:FBgn0038320 /sym=CG4931 /name= /prod= /func=
30	144882_at	1.559	FB:FBgn0030310 /sym=CG11709 /name= /prod=peptidoglycan recognition protein-like /func=defense/immunity protein
31	151778_at	1.554	FB:FBgn0031514 /sym=CG3332 /name= /prod= /func=
32	154077_at	1.546	FB:FBgn0032501 /sym=CG6263 /name= /prod=P-type ATPase /func=transporter
33	151554_at	1.546	BDGP:GH231.3prime-hit /ESTpos=maps 3prime of FB:FBgn0039026 /sym=CG7029 /name= /prod= /func=motor
34	151609_at	1.538	BDGP:LD1876.3prime-hit /ESTpos=maps 3prime of FB:FBgn0038501 /sym=CG5319 /name= /prod= /func=
35	141634_at	1.532	FB:FBgn0015075 /sym=Ddx1 /name=Dead-box-1 /prod= /func=ATP dependent helicase
36	142867_at	1.53	FB:FBgn0038666 /sym=CG5451 /name= /prod= /func=enzyme
37	148876_at	1.503	FB:FBgn0036603 /sym=CG13062 /name= /prod= /func=

Normal food vs. 0.5mM Cu, genes upregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold upregulation	gene
1	143277_at	7.986	FB:FBgn0002869 /sym=MtnB /name=Metallothionein B /prod=metallothionein B /func=Cu/Cd binding
2	147546_at	6.002	FB:FBgn0034511 /sym=CG13422 /name= /prod=gram-negative binding protein /func=defense/immunity protein
3	150257_at	5.95	FB:FBgn0038790 /sym=CG5097 /name= /prod=metallothionein /func=ligand binding or carrier
4	143276_at	4.032	FB:FBgn0002868 /sym=MtnA /name=Metallothionein A /prod=metallothionein A /func=Cu/Cd binding
5	141374_at	3.326	FB:FBgn0012042 /sym=AttA /name=Attacin-A /prod=attacin /func=antibacterial response protein
6	147220_s_at	2.769	FB:FBgn0033959 /sym=CG18372 /name= /prod= /func=
7	155082_at	1.799	FB:FBgn0001086 /sym=fzy /name=fizzy /prod= /func=enzyme
8	142349_at	1.776	FB:FBgn0038104 /sym=CG18550 /name= /prod= /func=
9	144899_at	1.7	FB:FBgn0030341 /sym=CG1967 /name= /prod= /func=
10	147569_at	1.653	FB:FBgn0034543 /sym=CG10404 /name= /prod= /func=
11	150180_at	1.62	FB:FBgn0038680 /sym=Cyp12a5 /name= /prod=cytochrome P450, CYP12A5 /func=cytochrome P45

Normal food vs. 5mM Zn, genes upregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold upregulation	gene
1	150257_at	16.58	FB:FBgn0038790 /sym=CG5097 /name= /prod=metallothionein /func=ligand binding or carrier
2	149759_at	12.49	FB:FBgn0038024 /sym=CG12242 /name= /prod= /func=
3	143277_at	8.669	FB:FBgn0002869 /sym=MtnB /name=Metallothionein B /prod=metallothionein B /func=Cu/Cd binding
4	147334_at	7.257	FB:FBgn0034160 /sym=CG5550 /name= /prod=restrictin-like /func=structural protein
5	152218_at	6.136	FB:FBgn0033783 /sym=CG17019 /name= /prod= /func=apoptosis inhibitor
6	147616_at	5.429	FB:FBgn0034612 /sym=CG10505 /name= /prod=ATP-binding cassette transporter /func=ion channel
7	148747_at	5.019	FB:FBgn0036419 /sym=CG13482 /name= /prod= /func=
8	152056_at	4.126	FB:FBgn0003231 /sym=ref(2)P /name=refractory to sigma P /prod= /func=
9	145977_at	4.072	FB:FBgn0031939 /sym=CG13796 /name= /prod= /func=neurotransmitter transporter
10	146815_at	4.044	FB:FBgn0033302 /sym=Cyp6a14 /name= /prod=cytochrome P450, CYP6A14 /func=cytochrome P45
11	142386_at	4.016	FB:FBgn0034247 /sym=CG6484 /name= /prod=sugar transporter /func=transporter
12	144846_at	3.945	FB:FBgn0030262 /sym=CG2081 /name= /prod= /func=
13	152096_at	3.68	FB:FBgn0036367 /sym=CG10116 /name= /prod=lipoprotein lipase-like /func=enzyme
14	146978_s_at	3.663	FB:FBgn0033576 /sym=CG18240 /name= /prod= /func=
15	143869_at	3.44	FB:FBgn0016076 /sym=vri /name=vrille /prod= /func=RNA polymerase II transcription factor
16	149799_at	3.351	FB:FBgn0038083 /sym=CG5999 /name= /prod=antennal-enriched UDP-glucuronosyltransferase-like /func=enzyme
17	141701_at	3.342	FB:FBgn0033205 /sym=CG2064 /name= /prod= /func=enzyme
18	143152_at	3.28	FB:FBgn0000594 /sym=Est-P /name=Esterase P /prod=carboxylesterase /func=carboxyesterase
19	149756_at	3.255	FB:FBgn0038021 /sym=CG4181 /name= /prod= /func=
20	151340_at	3.248	FB:FBgn0040867 /sym=CG11065 /name= /prod= /func=
21	150685_at	3.173	FB:FBgn0039455 /sym=CG14255 /name= /prod= /func=
22	152303_at	3.114	FB:FBgn0030183 /sym=CG15309 /name= /prod= /func=ligand binding or carrier
23	147043_at	3.113	FB:FBgn0033659 /sym=CG18188 /name=Death Associated Molecule related to Mch2 /prod=caspase /func=caspase
24	143784_at	3.07	FB:FBgn0015221 /sym=Fer2LCH /name=Ferritin 2 light chain homologue /prod=ferritin 2light chain-like /func=ferrous iron binding
25	152531_at	3.029	FB:FBgn0013307 /sym=Odc1 /name=Ornithine decarboxylase 1 /prod=ornithine decarboxylase /func=ornithine decarboxylase
26	143276_at	3.001	FB:FBgn0002868 /sym=MtnA /name=Metallothionein A /prod=metallothionein A /func=Cu/Cd binding
27	148401_at	2.976	FB:FBgn0035887 /sym=CG7170 /name= /prod= /func=endopeptidase
28	147436_at	2.852	FB:FBgn0034337 /sym=CG17524 /name= /prod=glutathione transferase /func=enzyme
29	150306_at	2.851	FB:FBgn0038873 /sym=CG5892 /name= /prod=O-antigen acetylase-like /func=enzyme
30	141416_at	2.825	FB:FBgn0036831 /sym=CG6839 /name= /prod=deoxyribonuclease I /func=enzyme
31	146742_at	2.82	FB:FBgn0033173 /sym=CG1645 /name= /prod= /func=
32	142878_at	2.813	FB:FBgn0030932 /sym=CG6461 /name= /prod=gamma-glutamyl transferase /func=enzyme
33	154237_at	2.812	FB:FBgn0030114 /sym=CG17754 /name= /prod= /func=transcription factor
34	148769_at	2.794	FB:FBgn0036454 /sym=CG17839 /name= /prod= /func=cell adhesion
35	148402_at	2.786	FB:FBgn0035888 /sym=CG7120 /name= /prod= /func=
36	149433_at	2.74	FB:FBgn0037487 /sym=CG14608 /name= /prod= /func=transmembrane receptor
37	149528_at	2.696	FB:FBgn0037635 /sym=CG9837 /name= /prod= /func=
38	153432_at	2.689	FB:FBgn0022073 /sym=Phas1 /name= /prod=eukaryotic-initiation-factor-4E binding/func=eukaryotic initiation factor 4E binding
39	152159_at	2.644	FB:FBgn0039856 /sym=CG1774 /name= /prod= /func=
40	146183_at	2.592	FB:FBgn0032284 /sym=CG7294 /name= /prod= /func=
41	145190_at	2.576	FB:FBgn0030756 /sym=CG9903 /name= /prod=sodium/bile acid symporter-like /func=transporter
42	144063_at	2.558	FB:FBgn0025456 /sym=CREG /name=Cellular Repressor of E1A-stimulated Genes /prod= /func=
43	149125_at	2.542	FB:FBgn0036985 /sym=CG5847 /name= /prod= /func=structural protein
44	152684_at	2.515	FB:FBgn0032358 /sym=CG4851 /name= /prod= /func=
45	148622_at	2.509	FB:FBgn0036229 /sym=CG7248 /name= /prod=peritrophin-like /func=ligand binding or carrier
46	148640_at	2.465	FB:FBgn0036264 /sym=CG11529 /name= /prod=trypsin-like /func=endopeptidase
47	152063_at	2.453	FB:FBgn0033820 /sym=CG4716 /name= /prod= /func=
48	152122_at	2.439	FB:FBgn0034296 /sym=CG10912 /name= /prod= /func=
49	151904_at	2.43	FB:FBgn0027598 /sym=BcDNA:GH03163 /name= /prod= /func=
50	154208_at	2.427	FB:FBgn0029820 /sym=CG16721 /name= /prod= /func=
51	151665_at	2.409	BDGP:LD25748.3prime-hit /ESTpos=maps 3prime of FB:FBgn0029903 /sym=CG4532 /name= /prod= /func=chaperone
52	148410_at	2.408	FB:FBgn0035904 /sym=CG6776 /name= /prod=glutathione transferase /func=glutathione transferase

53	152540_at	2.404	FB:FBgn0031248 /sym=CG11912 /name= /prod=mid-gut serine protease-like /func=endopeptidase
54	145121_at	2.394	FB:FBgn0030641 /sym=CG6299 /name= /prod= /func=enzyme
55	142730_at	2.392	FB:FBgn0029903 /sym=CG4532 /name= /prod= /func=chaperone
56	142419_at	2.384	FB:FBgn0030098 /sym=CG12057 /name= /prod= /func=
57	152525_at	2.356	FB:FBgn0010460 /sym=bun /name=bunched /prod= /func=RNA polymerase II transcription factor
58	153697_at	2.354	FB:FBgn0028717 /sym=Lnk /name= /prod= /func=cell cycle regulator
59	146832_at	2.35	FB:FBgn0033328 /sym=CG14745 /name= /prod=peptidoglycan recognition protein /func=defense/immunity protein
60	154909_at	2.349	FB:FBgn0005278 /sym=M(2)21AB /name=Minute (2) 21AB /prod=/func=methionine adenosyltransferase
61	148394_at	2.339	FB:FBgn0035877 /sym=CG7083 /name= /prod= /func=
62	143022_at	2.337	FB:FBgn0038376 /sym=CG4225 /name= /prod=ATP-binding cassette transporter /func=ion channel
63	146588_at	2.337	FB:FBgn0032948 /sym=CG17570 /name= /prod= /func=
64	146946_s_at	2.334	FB:FBgn0033518 /sym=CG11765 /name= /prod=antioxidant /func=enzyme
65	142737_at	2.321	FB:FBgn0037007 /sym=CG5059 /name= /prod= /func=
66	143385_at	2.303	FB:FBgn0003863 /sym=alphaTry /name=alphaTrypsin /prod=trypsin /func=trypsin
67	154485_at	2.288	FB:FBgn0001301 /sym=kel /name=kelch /prod= /func=transcription factor
68	154184_at	2.285	FB:FBgn0023129 /sym=aay /name=astray /prod=phosphoserine phosphatase-like /func=phosphoserine phosphatase
69	144521_at	2.268	FB:FBgn0029766 /sym=CG15784 /name= /prod= /func=
70	151955_at	2.257	FB:FBgn0000165 /sym=Bc /name=Black cells /prod=monophenol oxidase /func=monophenol monooxygenase
71	141444_at	2.253	FB:FBgn0030357 /sym=CG2471 /name= /prod= /func=actin binding
72	152140_at	2.248	FB:FBgn0032935 /sym=CG8678 /name= /prod= /func=enzyme
73	143486_at	2.246	FB:FBgn0004577 /sym=Pxd /name=Peroxidase /prod=peroxidase /func=peroxidase
74	146464_at	2.239	FB:FBgn0032733 /sym=CG15170 /name= /prod= /func=
75	142746_at	2.237	FB:FBgn0038081 /sym=CG10120 /name= /prod= /func=
76	143068_at	2.222	FB:FBgn0000078 /sym=Amy-d /name=Amylase distal /prod=alpha-amylase /func=alpha-amylase
77	149964_at	2.221	FB:FBgn0038346 /sym=CG14872 /name= /prod= /func=
78	151019_at	2.22	FB:FBgn0040532 /sym=CG8369 /name= /prod= /func=
79	152918_at	2.22	FB:FBgn0037308 /sym=CG1114 /name= /prod= /func=
80	143470_at	2.216	FB:FBgn0004431 /sym=LysX /name=Lysozyme X /prod=lysozyme X /func=lysozyme
81	147024_at	2.212	FB:FBgn0033639 /sym=CG9003 /name= /prod= /func=DNA binding
82	147422_at	2.199	FB:FBgn0034318 /sym=CG14500 /name= /prod= /func=ligand binding or carrier
83	152669_at	2.179	FB:FBgn0020385 /sym=Pug /name=pugilist /prod= /func=formate--tetrahydrofolate ligase
84	147696_at	2.173	FB:FBgn0034758 /sym=CG13510 /name= /prod= /func=
85	148678_at	2.166	FB:FBgn0036321 /sym=CG14120 /name= /prod= /func=
86	154884_at	2.163	FB:FBgn0035763 /sym=CG8602 /name= /prod=permease-like /func=transporter
87	151496_s_at	2.152	BDGP:GH7188.complete-hit /ESTpos=maps 3prime of FB:FBgn0039613 /sym=CG14527 /name= /prod= /func=endopeptidase
88	144227_at	2.144	FB:FBgn0028526 /sym=BG:DS01759.2 /name= /prod= /func=
89	143147_at	2.137	FB:FBgn0000565 /sym=Eip71CD /name=Ecdysone-induced protein 28/29kD /prod=/func=protein-methionine-S-oxide reductase
90	153165_at	2.127	FB:FBgn0035533 /sym=CG15015 /name= /prod= /func=cell cycle regulator
91	146133_at	2.118	FB:FBgn0032202 /sym=CG18619 /name= /prod= /func=
92	150456_at	2.117	FB:FBgn0039091 /sym=CG10182 /name= /prod= /func=
93	152146_at	2.111	FB:FBgn0020372 /sym=TM4SF /name=Transmembrane 4 superfamily /prod= /func=
94	142639_at	2.104	FB:FBgn0038809 /sym=CG16953 /name= /prod= /func=
95	145031_at	2.097	FB:FBgn0030520 /sym=CG10990 /name= /prod= /func=
96	142416_at	2.095	FB:FBgn0033779 /sym=CG3814 /name= /prod= /func=
97	142143_at	2.094	FB:FBgn0016078 /sym=wun /name=wunen /prod=phosphatidate phosphatase, type 2 /func=phosphatidate phosphatase
98	154077_at	2.093	FB:FBgn0032501 /sym=CG6263 /name= /prod=P-type ATPase /func=transporter
99	147569_at	2.089	FB:FBgn0034543 /sym=CG10404 /name= /prod= /func=
100	141734_at	2.086	FB:FBgn0030808 /sym=CG4937 /name= /prod= /func=signal transduction
101	151806_at	2.086	FB:FBgn0036732 /sym=CG7571 /name= /prod=organic anion transporter /func=transporter
102	152120_at	2.084	FB:FBgn0038194 /sym=Cyp6d5 /name= /prod=cytochrome P450, CYP6D5 /func=cytochrome P45
103	151794_at	2.083	FB:FBgn0028399 /sym=TMS1d /name= /prod= /func=
104	155157_at	2.078	FB:FBgn0039858 /sym=CG11525 /name= /prod= /func=cell cycle regulator
105	148390_at	2.074	FB:FBgn0035868 /sym=CG7194 /name= /prod= /func=
106	147492_at	2.072	FB:FBgn0034437 /sym=CG10051 /name= /prod= /func=
107	141460_at	2.067	FB:FBgn0031011 /sym=CG8034 /name= /prod=monocarboxylic acid transporter-like /func=transporter
108	143828_at	2.058	FB:FBgn0015589 /sym=Apc /name=APC-like /prod= /func=beta-catenin binding
109	143441_at	2.039	FB:FBgn0004228 /sym=mex1 /name=midgut expression 1 /prod=malic enzyme modifier /func=
110	153559_at	2.014	FB:FBgn0038729 /sym=CG7500 /name= /prod= /func=

111	152675_at	1.996	FB:FBgn0030484 /sym=CG1681 /name= /prod=glutathione transferase-like /func=enzyme
112	154124_at	1.993	FB:FBgn0035432 /sym=CG17723 /name= /prod=zinc transporter-like /func=transporter
113	151741_at	1.989	BDGP:SD2228.3prime-hit /ESTpos=maps 3prime of FB:FBgn0033198 /sym=CG2080 /name= /prod= /func=
114	153019_at	1.975	FB:FBgn0031294 /sym=CG4355 /name= /prod=protein tyrosine phosphatase-like /func=protein phosphatase
115	154514_at	1.965	FB:FBgn0025865 /sym=Cortactin /name=Cortactin /prod=cortactin /func=cell cycle regulator
116	154321_at	1.964	FB:FBgn0015222 /sym=Fer1HCH /name=Ferritin 1 heavy chain homologue/prod=ferritin 1heavy chain-like/func=ferrous ion binding
117	141522_at	1.957	FB:FBgn0033311 /sym=CG8643 /name= /prod= /func=nucleic acid binding
118	142220_at	1.956	FB:FBgn0033367 /sym=CG8193 /name= /prod=monophenol oxidase /func=enzyme
119	141732_at	1.952	FB:FBgn0004210 /sym=puc /name=puckered /prod=protein phosphatase /func=JUN kinase phosphatase
120	154247_at	1.948	FB:FBgn0034961 /sym=CG3163 /name= /prod= /func=
121	149552_s_at	1.925	FB:FBgn0037682 /sym=CG8125 /name= /prod= /func=aryldialkylphosphatase
122	151996_at	1.924	FB:FBgn0034797 /sym=CG12781 /name= /prod= /func=protein kinase
123	154110_at	1.921	FB:FBgn0035811 /sym=CG12262 /name= /prod= /func=enzyme
124	151038_at	1.912	FB:FBgn0040551 /sym=CG11686 /name= /prod= /func=
125	147615_at	1.912	FB:FBgn0034611 /sym=CG10069 /name= /prod=glycerol-3-phosphate transporter-like /func=transporter
126	146288_at	1.903	FB:FBgn0032435 /sym=CG6417 /name= /prod=organic anion transporter /func=transporter
127	149768_s_at	1.893	FB:FBgn0038034 /sym=Cyp9f3 /name= /prod= /func=cytochrome P45
128	141581_at	1.892	FB:FBgn0034063 /sym=CG8389 /name= /prod=monocarboxylate transporter-like /func=transporter
129	153065_at	1.891	FB:FBgn0028582 /sym=lqf /name=liquid facets /prod=epsin /func=
130	150180_at	1.89	FB:FBgn0038680 /sym=Cyp12a5 /name= /prod=cytochrome P450, CYP12A5 /func=cytochrome P45
131	149794_at	1.887	FB:FBgn0038074 /sym=CG6188 /name= /prod= /func=
132	154189_at	1.885	FB:FBgn0037084 /sym=CG5081 /name= /prod= /func=transporter
133	146788_at	1.88	FB:FBgn0033259 /sym=CG11210 /name= /prod= /func=
134	152622_at	1.875	FB:FBgn0037518 /sym=CG2641 /name= /prod= /func=
135	150708_at	1.873	FB:FBgn0039482 /sym=CG14258 /name= /prod= /func=
136	153434_at	1.872	FB:FBgn0001208 /sym=Hn /name=Henna /prod=phenylalanine 4-monooxygenase /func=phenylalanine 4-monooxygenase
137	145307_at	1.86	FB:FBgn0030929 /sym=CG15043 /name= /prod= /func=
138	148540_at	1.857	FB:FBgn0036111 /sym=CG6391 /name= /prod=diphosphoinositol polyphosphate phosphohydrolase /func=enzyme
139	154259_at	1.854	FB:FBgn0031846 /sym=CG11331 /name= /prod= /func=
140	154214_at	1.842	FB:FBgn0035028 /sym=CG3522 /name= /prod=cholesterol transfer protein-like /func=transporter
141	152274_at	1.842	FB:FBgn0032735 /sym=CG10650 /name= /prod= /func=endopeptidase
142	145681_at	1.841	FB:FBgn0031477 /sym=CG3098 /name= /prod=interferon-like /func=signal transduction
143	149052_at	1.831	FB:FBgn0036875 /sym=CG9449 /name= /prod=acid phosphatase-like /func=enzyme
144	146613_at	1.83	FB:FBgn0032979 /sym=CG1832 /name= /prod= /func=nucleic acid binding
145	142226_at	1.823	FB:FBgn0000416 /sym=Sap-r /name=Saposin-related /prod=prosaposin-like /func=
146	141588_at	1.823	FB:FBgn0003742 /sym=tra2 /name=transformer 2 /prod= /func=RNA binding
147	145249_at	1.821	FB:FBgn0030847 /sym=CG12991 /name= /prod= /func=
148	151801_at	1.817	FB:FBgn0037303 /sym=CG12163 /name= /prod=cathepsin L-like /func=endopeptidase
149	152434_at	1.815	FB:FBgn0039825 /sym=CG12074 /name= /prod= /func=ligand binding or carrier
150	143035_at	1.815	FB:FBgn0038745 /sym=CG4538 /name= /prod= /func=endopeptidase
151	147285_at	1.807	FB:FBgn0034085 /sym=CG18243 /name= /prod= /func=
152	148484_at	1.807	FB:FBgn0036030 /sym=CG6767 /name= /prod= /func=ribose-phosphate pyrophosphokinase
153	141842_at	1.803	BDGP:GH06241.3prime-hit /maps to FB:FBgn0034404 (/sym=CG15101 /name= /prod=/func=
154	152070_at	1.802	FB:FBgn0030521 /sym=CG10992 /name= /prod=cathepsin B /func=endopeptidase
155	146406_at	1.802	FB:FBgn0032639 /sym=CG18563 /name= /prod= /func=
156	153798_at	1.796	FB:FBgn0035715 /sym=CG10103 /name= /prod= /func=transcription factor
157	154112_at	1.796	FB:FBgn0038220 /sym=CG12207 /name= /prod= /func=
158	153560_at	1.795	FB:FBgn0030229 /sym=CG1534 /name= /prod= /func=
159	141682_at	1.791	FB:FBgn0033227 /sym=CG1548 /name= /prod= /func=
160	143797_at	1.789	FB:FBgn0015320 /sym=UbcD2 /name=Ubiquitin conjugating enzyme 2 /prod=/func=ubiquitin conjugating enzyme
161	145357_at	1.789	FB:FBgn0031000 /sym=CG7876 /name= /prod=salivary glue protein /func=structural protein
162	145637_at	1.787	FB:FBgn0031418 /sym=CG3609 /name= /prod= /func=transcription factor binding
163	149505_at	1.784	FB:FBgn0037593 /sym=CG11740 /name= /prod= /func=
164	143802_at	1.781	FB:FBgn0015351 /sym=CG14906 /name= /prod= /func=
165	149976_at	1.777	FB:FBgn0038366 /sym=CG4576 /name= /prod= /func=
166	153850_at	1.777	FB:FBgn0038100 /sym=CG12358 /name= /prod= /func=
167	154786_at	1.775	FB:FBgn0038347 /sym=CG18522 /name= /prod= /func=
168	153045_at	1.771	FB:FBgn0037252 /sym=CG14650 /name= /prod= /func=chaperone

169	152656_at	1.771	FB:FBgn0020304 /sym=drongo /name=drongo /prod= /func=defense/immunity protein
170	141349_at	1.769	FB:FBgn0036945 /sym=CG6981 /name= /prod= /func=
171	153728_at	1.767	FB:FBgn0012037 /sym=Ance /name=Angiotensin converting enzyme/prod=angiotensin I-converting enz/func=peptidyl-dipeptidase
172	153769_at	1.767	FB:FBgn0012034 /sym=AcCoAS /name=Acetyl Coenzyme A synthase /prod=acetate--CoA ligase /func=acetate--CoA ligase
173	146993_at	1.766	FB:FBgn0033595 /sym=CG18337 /name= /prod= /func=
174	141285_at	1.766	FB:FBgn0038320 /sym=CG4931 /name= /prod= /func=
175	153028_at	1.762	FB:FBgn0014184 /sym=guf /name=gut feeling /prod=ornithine decarboxylase antienzyme /func=ornithine decarboxylase inhibitor
176	146771_at	1.756	FB:FBgn0033226 /sym=CG1882 /name= /prod= /func=enzyme
177	141687_at	1.755	FB:FBgn0003507 /sym=srp /name=serpent /prod=GATA factor /func=general RNA polymerase II transcription factor
178	145185_at	1.752	FB:FBgn0030747 /sym=CG4301 /name= /prod=P-type ATPase /func=transporter
179	142992_at	1.752	FB:FBgn0035236 /sym=CG12004 /name= /prod= /func=
180	145265_at	1.751	FB:FBgn0030876 /sym=CG6762 /name= /prod= /func=
181	141233_at	1.749	FB:FBgn0029831 /sym=CG5966 /name= /prod=triacylglycerol lipase /func=enzyme
182	151931_at	1.749	FB:FBgn0015795 /sym=Rab7 /name=Rab-protein 7 /prod= /func=RHO small GTPase
183	141408_at	1.745	FB:FBgn0038416 /sym=CG17930 /name= /prod=sugar transporter-like /func=transporter
184	152927_at	1.743	FB:FBgn0030398 /sym=CG2555 /name= /prod=larval cuticle protein-like /func=structural protein
185	152306_at	1.739	FB:FBgn0030531 /sym=CG11058 /name= /prod= /func=enzyme
186	143599_at	1.736	FB:FBgn0010341 /sym=Cdc42 /name=Cdc42 /prod= /func=RHO small GTPase
187	152456_at	1.736	FB:FBgn0019643 /sym=Dat /name=Dopamine N acetyltransferase /prod=/func=arylalkylamine N-acetyltransferase
188	144790_at	1.736	FB:FBgn0030189 /sym=CG2909 /name= /prod= /func=
189	147124_at	1.73	FB:FBgn0033786 /sym=CG3884 /name= /prod= /func=
190	142690_at	1.727	FB:FBgn0035371 /sym=CG9977 /name= /prod=adenosylhomocysteinase /func=enzyme
191	148450_at	1.726	FB:FBgn0035968 /sym=CG4484 /name= /prod=hydrogen/sucrose transporter-like /func=transporter
192	151810_at	1.722	FB:FBgn0033179 /sym=CG11139 /name= /prod= /func=
193	147469_at	1.719	FB:FBgn0034398 /sym=CG15098 /name= /prod= /func=
194	141670_at	1.718	FB:FBgn0033901 /sym=CG12366 /name= /prod= /func=
195	142822_at	1.715	FB:FBgn0032727 /sym=CG10623 /name= /prod= /func=
196	147108_at	1.715	FB:FBgn0033760 /sym=CG8785 /name= /prod=amino-acid permease-like /func=transporter
197	148265_at	1.714	FB:FBgn0035679 /sym=CG10467 /name= /prod=aldose 1-epimerase-like /func=enzyme
198	143861_at	1.712	FB:FBgn0015933 /sym=didum /name=dilute class unconventional myosin /prod=myosin class V /func=actin binding
199	144243_at	1.71	FB:FBgn0028679 /sym=Sema-5c /name= /prod=semaphorin /func=transmembrane receptor
200	143742_at	1.704	FB:FBgn0014010 /sym=Rab5 /name=Rab-protein 5 /prod= /func=RAB small GTPase
201	152058_at	1.699	FB:FBgn0003261 /sym=Rm62 /name=Rm62 /prod= /func=ATP dependent RNA helicase
202	143218_at	1.694	FB:FBgn0001977 /sym=l(2)35Bg /name=lethal (2) 35Bg /prod= /func=
203	153701_at	1.693	FB:FBgn0001980 /sym=gft /name=guftagu /prod=cullin /func=cell cycle regulator
204	154332_at	1.691	FB:FBgn0039541 /sym=CG12876 /name= /prod= /func=enzyme
205	153730_at	1.691	FB:FBgn0033551 /sym=CG7222 /name= /prod= /func=
206	143681_at	1.688	FB:FBgn0011710 /sym=36770 /name=Septin-1 /prod=septin /func=GTP binding
207	153013_at	1.688	FB:FBgn0028916 /sym=BG:DS01068.2 /name= /prod= /func=
208	151323_at	1.687	FB:FBgn0040850 /sym=CG15210 /name= /prod= /func=
209	147319_at	1.685	FB:FBgn0034140 /sym=CG8317 /name= /prod= /func=
210	149169_at	1.684	FB:FBgn0037065 /sym=CG12974 /name= /prod= /func=
211	152692_at	1.683	FB:FBgn0027621 /sym=6-phosphofructo-2-kinase /name=6-phosphofructo-2-kinase /prod=/func=6-phosphofructo-2-kinase
212	154742_at	1.683	FB:FBgn0011744 /sym=Arp66B /name=Actin-related protein 66B /prod=actin-like protein /func=cytoskeletal structural protein
213	151972_at	1.68	FB:FBgn0030679 /sym=CG8206 /name= /prod= /func=
214	143145_at	1.679	FB:FBgn0000562 /sym=egl /name=egalitarian /prod= /func=structural protein
215	152298_at	1.676	FB:FBgn0037343 /sym=CG1081 /name= /prod= /func=RHEB small monomeric GTPase ; EC:3.6.1.47 | from sequence similarity
216	145850_i_at	1.673	FB:FBgn0031744 /sym=CG14002 /name= /prod= /func= /map=26A4-26A4 /transc=CT33558 /len=141 /GB:AE003611
217	143104_at	1.669	FB:FBgn0000339 /sym=cni /name=cornichon /prod= /func= /map=35F8-35F8 /transc=CT18361 /len=836 /GB:AE003650
218	148469_at	1.668	FB:FBgn0036007 /sym=CG3424 /name= /prod=amino-acid permease-like /func=transporter
219	148839_at	1.665	FB:FBgn0036556 /sym=CG5830 /name= /prod= /func=
220	145744_at	1.664	FB:FBgn0031580 /sym=CG15423 /name= /prod= /func=
221	145743_at	1.664	FB:FBgn0031579 /sym=CG15422 /name= /prod= /func=
222	154640_at	1.663	FB:FBgn0037116 /sym=CG7158 /name= /prod= /func=1-phosphatidylinositol-4-phosphate kinase
223	142605_at	1.658	FB:FBgn0039276 /sym=CG11938 /name= /prod= /func=
224	144148_at	1.657	FB:FBgn0026565 /sym=BG:DS00004.14 /name= /prod=argininosuccinate synthase /func=arginosuccinate synthase
225	151387_at	1.655	FB:FBgn0040918 /sym=CG15898 /name= /prod= /func=
226	152507_at	1.651	FB:FBgn0003317 /sym=sax /name=saxophone /prod=/func=type I transforming growth factor beta receptor

227	146833_at	1.649	FB:FBgn0033329 /sym=CG8575 /name= /prod= /func=
228	155139_at	1.645	FB:FBgn0036837 /sym=CG18135 /name= /prod= /func=
229	153665_at	1.639	FB:FBgn0017558 /sym=Pdk /name=Pyruvate dehydrogenase kinase /prod=/func=pyruvate dehydrogenase (lipoamide) kinase
230	153725_at	1.637	FB:FBgn0031285 /sym=CG3662 /name= /prod= /func=structural protein
231	153287_at	1.636	FB:FBgn0023528 /sym=EG:25E8.2 /name= /prod=ubiquitin conjugating enzyme-like /func=ubiquitin conjugating enzyme
232	147003_at	1.631	FB:FBgn0033605 /sym=CG9067 /name= /prod= /func=
233	153218_at	1.63	FB:FBgn0034709 /sym=CG3074 /name= /prod= /func=endopeptidase
234	152687_at	1.629	FB:FBgn0030688 /sym=CG8952 /name= /prod=trypsin-like /func=serine-type endopeptidase
235	145745_at	1.622	FB:FBgn0031581 /sym=CG10039 /name= /prod= /func=
236	153384_at	1.619	FB:FBgn0031695 /sym=Cyp4ac3 /name= /prod=cytochrome P450, CYP4AC3 /func=cytochrome P45
237	148016_at	1.618	FB:FBgn0035296 /sym=CG11814 /name= /prod= /func=
238	153232_at	1.613	FB:FBgn0032121 /sym=CG18419 /name= /prod=P-type ATPase /func=transporter
239	146758_at	1.611	FB:FBgn0033207 /sym=CG12826 /name= /prod= /func=
240	152830_at	1.608	FB:FBgn0035154 /sym=CG3344 /name= /prod= /func=peptidase
241	146718_at	1.606	FB:FBgn0033139 /sym=CG12837 /name= /prod= /func=
242	143535_at	1.605	FB:FBgn0004914 /sym=Hnf4 /name=Hepatocyte nuclear factor 4 /prod=hepatocyte nf 4 /func=ligand-dependent nuclear receptor
243	154256_at	1.604	FB:FBgn0000173 /sym=ben /name=bendless /prod=ubiquitin conjugating enzyme /func=ubiquitin conjugating enzyme
244	142794_at	1.604	FB:FBgn0017581 /sym=Lk6 /name=heat shock construct of Kidd /prod=/func=protein serine/threonine kinase
245	151117_at	1.599	FB:FBgn0040632 /sym=CG5145 /name= /prod= /func=
246	154757_at	1.598	FB:FBgn0035498 /sym=CG14991 /name= /prod=mitogen inducible protein-like /func=
247	153903_at	1.597	FB:FBgn0028691 /sym=Rpn4 /name= /prod=19S proteasome regulatory particle, non-ATPase, subunit S13 /func=endopeptidase
248	147276_at	1.596	FB:FBgn0034071 /sym=CG8405 /name= /prod= /func=
249	152311_at	1.596	FB:FBgn0037049 /sym=CG10577 /name= /prod= /func=enzyme
250	152267_at	1.595	FB:FBgn0011576 /sym=Cyp4d2 /name=Cytochrome P45-4d2 /prod=cytochrome P450, CYP4D2 /func=cytochrome P45
251	151742_at	1.594	BDGP:SD3655.3prime-hit /ESTpos=maps 3prime of FB:FBgn0035236 /sym=CG12004 /name= /prod= /func=
252	147105_at	1.585	FB:FBgn0033755 /sym=CG8594 /name= /prod=chloride channel /func=ion channel
253	151494_at	1.585	BDGP:GH6422.3prime-hit /ESTpos=maps 3prime of FB:FBgn0035115 /sym=CG13878 /name= /prod= /func=
254	152430_at	1.584	FB:FBgn0033780 /sym=CG3845 /name= /prod= /func=translation factor
255	146114_at	1.581	FB:FBgn0032170 /sym=CG4658 /name= /prod= /func=
256	151409_at	1.58	FB:FBgn0040942 /sym=CG12643 /name= /prod= /func=
257	152816_at	1.579	FB:FBgn0029990 /sym=CG2233 /name= /prod= /func=
258	144828_at	1.575	FB:FBgn0030236 /sym=CG1664 /name= /prod= /func=
259	153069_at	1.572	FB:FBgn0034394 /sym=CG15096 /name= /prod=sodium/phosphate cotransporter /func=transporter
260	144018_at	1.571	FB:FBgn0024361 /sym=EG:8D8.7 /name= /prod= /func=
261	153226_at	1.57	FB:FBgn0036508 /sym=CG7439 /name= /prod= /func=
262	150870_at	1.569	FB:FBgn0039742 /sym=CG15528 /name= /prod=protein phosphatase-like /func=protein phosphatase
263	152715_at	1.568	FB:FBgn0033188 /sym=CG1600 /name= /prod= /func=enzyme
264	143295_at	1.567	FB:FBgn0003046 /sym=Pcp /name=Pupal cuticle protein /prod= /func=structural protein of pupal cuticle (Drosophila)
265	146708_at	1.566	FB:FBgn0033125 /sym=CG12846 /name= /prod= /func=
266	145728_at	1.565	FB:FBgn0031558 /sym=CG16704 /name= /prod= /func=
267	142899_at	1.562	FB:FBgn0032197 /sym=CG5694 /name= /prod= /func=
268	142301_s_at	1.561	FB:FBgn0036172 /sym=CG11711 /name= /prod= /func=
269	145647_at	1.56	FB:FBgn0031432 /sym=Cyp309a1 /name= /prod=cytochrome P450, CYP309A1 /func=cytochrome P45
270	148527_at	1.559	FB:FBgn0036092 /sym=CG6491 /name= /prod= /func=
271	153493_at	1.558	FB:FBgn0034068 /sym=CG8400 /name= /prod= /func=ligand binding or carrier
272	153031_at	1.556	FB:FBgn0038037 /sym=Cyp9f2 /name= /prod=cytochrome P450, CYP9F2 /func=cytochrome P45
273	151556_s_at	1.556	BDGP:GH2347.3prime-hit /ESTpos=maps in FB:FBgn0028542 /sym=BG:DS00180.8 /name= /prod= /func=cell adhesion
274	155128_at	1.556	FB:FBgn0029941 /sym=CG1677 /name= /prod= /func=signal transduction
275	144581_at	1.549	FB:FBgn0029849 /sym=CG3774 /name= /prod=nucleotide-sugar transporter /func=transporter
276	153802_at	1.547	FB:FBgn0039132 /sym=CG5864 /name= /prod=clathrin adaptor protein /func=transporter
277	155131_at	1.546	FB:FBgn0037719 /sym=CG9424 /name= /prod= /func=
278	151851_at	1.546	FB:FBgn0027611 /sym=BcDNA:GH02419 /name= /prod=alpha-mannosidase /func=enzyme
279	151888_at	1.541	FB:FBgn0027930 /sym=BEST:GH02921 /name= /prod= /func=endopeptidase
280	142733_at	1.541	FB:FBgn0036948 /sym=CG7298 /name= /prod=peritrophin-like /func=ligand binding or carrier
281	147876_at	1.539	FB:FBgn0035040 /sym=CG4741 /name= /prod= /func=
282	144599_at	1.532	FB:FBgn0029875 /sym=CG3950 /name= /prod= /func=DNA binding
283	145847_at	1.529	FB:FBgn0031741 /sym=CG11034 /name= /prod=dipeptidyl-peptidase IV /func=peptidase
284	151499_at	1.528	BDGP:GH8192.3prime-hit /ESTpos=maps 3prime of FB:FBgn0029801 /sym=CG15771 /name= /prod= /func=

285	146272_at	1.527	FB:FBgn0032412 /sym=CG16996 /name= /prod= /func=endopeptidase
286	151711_at	1.526	BDGP:LD43519.3prime-hit /ESTpos=maps 3prime of FB:FBgn0035616 /sym=CG4867 /name= /prod= /func=
287	154034_at	1.521	FB:FBgn0037442 /sym=CG10277 /name= /prod= /func=
288	145187_at	1.521	FB:FBgn0030752 /sym=CG9947 /name= /prod= /func=
289	153615_at	1.517	FB:FBgn0030245 /sym=CG1637 /name= /prod= /func=acid phosphatase
290	143231_at	1.516	FB:FBgn0002525 /sym=Lam /name=Lamin /prod=lamin /func=cytoskeletal structural protein
291	149123_at	1.514	FB:FBgn0036983 /sym=CG5408 /name= /prod= /func=
292	154340_at	1.512	FB:FBgn0030986 /sym=CG7481 /name= /prod= /func=signal transduction
293	144536_at	1.512	FB:FBgn0029786 /sym=CG3171 /name= /prod= /func=
294	154701_at	1.512	FB:FBgn0029943 /sym=CG1643 /name= /prod= /func=
295	154804_at	1.512	FB:FBgn0033352 /sym=CG8232 /name= /prod=PAB-dependent poly(A)-specific ribonuclease subunit /func=enzyme
296	141762_at	1.51	FB:FBgn0013770 /sym=Cp1 /name=Cysteine proteinase-1 /prod=cathepsin L /func=cathepsin L
297	143944_at	1.508	FB:FBgn0020906 /sym=Ser4 /name=Serine protease 4 /prod=serine endopeptidase /func=serine-type endopeptidase
298	152337_at	1.508	FB:FBgn0029878 /sym=CG10695 /name= /prod= /func=motor
299	154725_at	1.503	FB:FBgn0004603 /sym=Src42A /name=Src oncogene at 42A /prod=protein tyrosine kinase /func=protein tyrosine kinase

Normal food vs. 0.5 mM BCS, genes upregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold upregulation	gene
1	147334_at	6.887	FB:FBgn0034160 /sym=CG5550 /name= /prod=restrictin-like /func=structural protein
2	149759_at	6.814	FB:FBgn0038024 /sym=CG12242 /name= /prod= /func=
3	148222_at	6.392	FB:FBgn0035607 /sym=CG4835 /name= /prod=peritrophin/chitinase /func=structural protein
4	150579_at	6.32	FB:FBgn0039316 /sym=CG11893 /name= /prod= /func=
5	148678_at	5.622	FB:FBgn0036321 /sym=CG14120 /name= /prod= /func=
6	152734_at	5.356	FB:FBgn0028940 /sym=Cyp28a5 /name= /prod=cytochrome P450, CYP28A5 /func=cytochrome P45
7	149799_at	4.775	FB:FBgn0038083 /sym=CG5999 /name= /prod=antennal-enriched UDP-glucuronosyltransferase-like /func=enzyme
8	149024_at	4.658	FB:FBgn0036833 /sym=CG3819 /name= /prod= /func=
9	149699_at	4.503	FB:FBgn0037934 /sym=CG6830 /name= /prod= /func=
10	147225_at	4.439	FB:FBgn0033978 /sym=Cyp6a23 /name= /prod=cytochrome P450, CYP6A23 /func=cytochrome P45
11	149489_at	4.33	FB:FBgn0037575 /sym=CG7459 /name= /prod=copper transporter-like /func=transporter
12	142386_at	4.322	FB:FBgn0034247 /sym=CG6484 /name= /prod=sugar transporter /func=transporter
13	149798_at	3.992	FB:FBgn0038082 /sym=CG5724 /name= /prod=antennal-enriched UDP-glucuronosyltransferase-like /func=enzyme
14	142733_at	3.775	FB:FBgn0036948 /sym=CG7298 /name= /prod=peritrophin-like /func=ligand binding or carrier
15	148747_at	3.771	FB:FBgn0036419 /sym=CG13482 /name= /prod= /func=
16	143385_at	3.72	FB:FBgn0003863 /sym=alphaTry /name=alphaTrypsin /prod=trypsin /func=trypsin
17	148289_at	3.526	FB:FBgn0035718 /sym=CG14820 /name= /prod=carboxypeptidase A-like /func=peptidase
18	146379_at	3.483	FB:FBgn0032606 /sym=CG17932 /name= /prod=UDP-glucuronosyltransferase /func=enzyme
19	147043_at	3.432	FB:FBgn0033659 /sym=CG18188 /name=Death Associated Molecule related to Mch2 /prod=caspase /func=caspase
20	152554_at	3.351	FB:FBgn0038899 /sym=CG5845 /name= /prod=membrane alanine aminopeptidase-like (inactive) /func=peptidase
21	142222_at	3.349	FB:FBgn0015039 /sym=Cyp9b2 /name=Cytochrome P45-9b2 /prod=cytochrome P450, CYP9B2 /func=cytochrome P45
22	144332_at	3.337	FB:FBgn0028950 /sym=BG:BACR44L22.1 /name= /prod=astacin-like /func=metalloendopeptidase
23	146262_at	3.284	FB:FBgn0032387 /sym=CG16965 /name= /prod= /func=enzyme
24	153019_at	3.276	FB:FBgn0031294 /sym=CG4355 /name= /prod=protein tyrosine phosphatase-like /func=protein phosphatase
25	141439_at	3.243	FB:FBgn0035326 /sym=CG13805 /name= /prod= /func=ligand binding or carrier
26	153088_at	3.211	FB:FBgn0035770 /sym=CG8588 /name= /prod= /func=
27	151899_at	3.174	FB:FBgn0036992 /sym=CG11796 /name= /prod= /func=
28	152122_at	3.088	FB:FBgn0034296 /sym=CG10912 /name= /prod= /func=
29	149964_at	3.013	FB:FBgn0038346 /sym=CG14872 /name= /prod= /func=
30	145449_at	2.753	FB:FBgn0031140 /sym=CG12092 /name= /prod= /func=transmembrane receptor
31	148622_at	2.741	FB:FBgn0036229 /sym=CG7248 /name= /prod=peritrophin-like /func=ligand binding or carrier
32	152006_at	2.738	FB:FBgn0035670 /sym=CG10472 /name= /prod=chymotrypsin-like serine protease /func=endopeptidase
33	141701_at	2.694	FB:FBgn0033205 /sym=CG2064 /name= /prod= /func=enzyme
34	153470_at	2.691	FB:FBgn0034436 /sym=CG11961 /name= /prod= /func=
35	142407_at	2.682	FB:FBgn0036622 /sym=CG4753 /name= /prod=1-acylglycerol-3-phosphate O-acyltransferase-like /func=enzyme
36	149873_at	2.671	FB:FBgn0038200 /sym=CG9920 /name= /prod=heat shock protein 1, 10 kD (chaperonin 10) /func=chaperone
37	150887_at	2.649	FB:FBgn0039768 /sym=CG15533 /name= /prod=sphingomyelin phosphodiesterase /func=enzyme
38	146742_at	2.595	FB:FBgn0033173 /sym=CG1645 /name= /prod= /func=
39	147422_at	2.56	FB:FBgn0034318 /sym=CG14500 /name= /prod= /func=ligand binding or carrier
40	153738_at	2.476	FB:FBgn0024947 /sym=NTPase /name= /prod= /func=enzyme
41	142561_at	2.469	FB:FBgn0039871 /sym=CG2245 /name= /prod= /func=
42	142201_at	2.448	FB:FBgn0034663 /sym=CG4363 /name= /prod= /func=
43	147108_at	2.44	FB:FBgn0033760 /sym=CG8785 /name= /prod=amino-acid permease-like /func=transporter
44	150582_at	2.436	FB:FBgn0039319 /sym=CG13659 /name= /prod= /func=
45	143695_at	2.427	FB:FBgn0011822 /sym=pcl /name=pepsinogen-like /prod=cathepsin E /func=cathepsin E
46	151808_at	2.423	FB:FBgn0035212 /sym=CG9165 /name= /prod=hydroxymethylbilane synthase /func=enzyme
47	152289_at	2.418	FB:FBgn0030148 /sym=CG3106 /name= /prod=transmembrane protein NRF-6 like /func=transmembrane receptor
48	147718_at	2.375	FB:FBgn0034783 /sym=CG9825 /name= /prod=sodium/phosphate cotransporter /func=transporter
49	142416_at	2.373	FB:FBgn0033779 /sym=CG3814 /name= /prod= /func=
50	149652_at	2.352	FB:FBgn0037846 /sym=CG6574 /name= /prod=reduced folate transporter-like /func=transporter
51	146826_at	2.347	FB:FBgn0033319 /sym=CG8579 /name= /prod=serine protease-like /func=endopeptidase
52	141390_at	2.286	FB:FBgn0033318 /sym=CG8732 /name= /prod=acetate--CoA ligase-like /func=enzyme

53	148677_at	2.251	FB:FBgn0036320 /sym=CG10943 /name= /prod= /func=motor
54	144331_at	2.23	FB:FBgn0028949 /sym=BG:BACR44L22.2 /name= /prod=astacin-like /func=metalloendopeptidase
55	146060_at	2.207	FB:FBgn0032087 /sym=CG9568 /name= /prod= /func=
56	141664_at	2.14	FB:FBgn0015245 /sym=Hsp60 /name=Heat shock protein 6 /prod=heat shock protein 60 kD /func=heat shock protein
57	146288_at	2.133	FB:FBgn0032435 /sym=CG6417 /name= /prod=organic anion transporter /func=transporter
58	141349_at	2.091	FB:FBgn0036945 /sym=CG6981 /name= /prod= /func=
59	149169_at	2.081	FB:FBgn0037065 /sym=CG12974 /name= /prod= /func=
60	153576_at	2.059	FB:FBgn0036182 /sym=CG6084 /name= /prod=aldehyde reductase /func=enzyme
61	149569_at	2.057	FB:FBgn0037715 /sym=CG9399 /name= /prod= /func=
62	145847_at	2.053	FB:FBgn0031741 /sym=CG11034 /name= /prod=dipeptidyl-peptidase IV /func=peptidase
63	145850_i_at	2.038	FB:FBgn0031744 /sym=CG14002 /name= /prod= /func=
64	144899_at	2.02	FB:FBgn0030341 /sym=CG1967 /name= /prod= /func=
65	143784_at	2.017	FB:FBgn0015221 /sym=Fer2LCH/name=Ferritin 2 light chain homologue/prod=ferritin 2light chain-like/func=ferrous ion binding
66	149479_at	2.013	FB:FBgn0037564 /sym=CG11673 /name= /prod= /func=
67	141409_at	2.008	FB:FBgn0039154 /sym=CG6164 /name= /prod= /func=
68	145796_at	2.001	FB:FBgn0031654 /sym=CG8869 /name= /prod=serine protease-like /func=endopeptidase
69	141416_at	11.89	FB:FBgn0036831 /sym=CG6839 /name= /prod=deoxyribonuclease I /func=enzyme
70	144287_at	1.957	FB:FBgn0028883 /sym=BG:DS04095.3 /name= /prod= /func=
71	145202_at	1.955	FB:FBgn0030775 /sym=CG9673 /name= /prod= /func=endopeptidase
72	142800_at	1.948	FB:FBgn0003557 /sym=Su(dx) /name=Suppressor of deltex /prod=ubiquitin--protein ligase /func=ubiquitin--protein ligase
73	145357_at	1.943	FB:FBgn0031000 /sym=CG7876 /name= /prod=salivary glue protein /func=structural protein
74	146958_at	1.909	FB:FBgn0033541 /sym=CG12934 /name= /prod= /func=
75	142340_at	1.907	FB:FBgn0035494 /sym=CG14993 /name= /prod=fumarylacetoacetase-like /func=enzyme
76	148087_at	1.905	FB:FBgn0035412 /sym=CG14957 /name= /prod= /func=
77	151960_at	1.903	FB:FBgn0010246 /sym=Myo61F /name=Myosin 61F /prod=myosin I heavy chain /func=actin binding
78	144521_at	1.899	FB:FBgn0029766 /sym=CG15784 /name= /prod= /func=
79	154318_at	1.896	FB:FBgn0033304 /sym=Cyp6a13 /name= /prod=cytochrome P450, CYP6A13 /func=cytochrome P45
80	148711_at	1.894	FB:FBgn0036361 /sym=CG10154 /name= /prod=peritrophin-like /func=structural protein
81	149833_at	1.877	FB:FBgn0038137 /sym=CG11688 /name= /prod= /func=peptidase
82	141288_at	1.877	FB:FBgn0037657 /sym=CG11990 /name= /prod= /func=
83	142331_i_at	1.877	FB:FBgn0034740 /sym=CG3875 /name= /prod= /func=RNA binding
84	144847_at	1.859	FB:FBgn0030264 /sym=CG1961 /name= /prod= /func=enzyme
85	154786_at	1.853	FB:FBgn0038347 /sym=CG18522 /name= /prod= /func=
86	143660_at	1.846	FB:FBgn0011555 /sym=thetaTry /name=thetaTrypsin /prod=trypsin /func=trypsin
87	141395_at	1.828	FB:FBgn0036362 /sym=CG10725 /name= /prod=peritrophin-like /func=structural protein
88	150340_at	1.828	FB:FBgn0038923 /sym=CG13410 /name= /prod= /func=
89	144654_at	1.796	FB:FBgn0029960 /sym=CG12157 /name= /prod= /func=
90	151838_at	1.792	FB:FBgn0031263 /sym=CG2789 /name= /prod=peripheral-type benzodiazepine receptor /func=receptor
91	143699_at	1.787	FB:FBgn0011834 /sym=Ser6 /name=Serine protease 6 /prod=serine carboxypeptidase /func=serine carboxypeptidase
92	152897_at	1.76	FB:FBgn0036422 /sym=CG3868 /name= /prod= /func=signal transduction
93	147410_at	1.748	FB:FBgn0034295 /sym=CG10911 /name= /prod= /func=
94	141259_at	1.742	FB:FBgn0030496 /sym=CG1733 /name= /prod= /func=
95	142756_at	1.734	FB:FBgn0030263 /sym=CG2076 /name= /prod= /func=
96	150180_at	1.732	FB:FBgn0038680 /sym=Cyp12a5 /name= /prod=cytochrome P450, CYP12A5 /func=cytochrome P45
97	145743_at	1.73	FB:FBgn0031579 /sym=CG15422 /name= /prod= /func=
98	152717_at	1.727	FB:FBgn0023507 /sym=EG:87B1.3 /name= /prod=actin binding protein /func=actin binding
99	142717_at	1.726	FB:FBgn0003741 /sym=tra /name=transformer /prod= /func=RNA binding
100	143245_at	1.723	FB:FBgn0002578 /sym=m1 /name=E(spl) region transcript m1 /prod= /func=
101	152312_at	1.719	FB:FBgn0034048 /sym=CG8256 /name= /prod=glycerol-3-phosphate dehydrogenase-like /func=enzyme
102	151932_at	1.719	FB:FBgn0000406 /sym=Cyt-b5 /name=Cytochrome b5-related /prod=cytochrome b5-like /func=electron transfer
103	148171_at	1.716	FB:FBgn0035535 /sym=CG15017 /name= /prod= /func=
104	142690_at	1.707	FB:FBgn0035371 /sym=CG9977 /name= /prod=adenosylhomocysteinase /func=enzyme
105	142807_at	1.694	FB:FBgn0040994 /sym=CG17567 /name= /prod= /func=
106	150825_at	1.689	FB:FBgn0039670 /sym=CG7567 /name= /prod= /func=
107	152233_at	1.673	FB:FBgn0027657 /sym=glob1 /name=globin 1 /prod=globin /func=ligand binding or carrier
108	144823_at	1.669	FB:FBgn0030227 /sym=CG9732 /name= /prod= /func=
109	143929_at	1.664	FB:FBgn0020508 /sym=Ag5r2 /name=Antigen 5-related 2 /prod= /func=
110	150265_at	1.661	FB:FBgn0038804 /sym=CG10877 /name= /prod= /func=alpha-methylacyl-CoA racemase

111	152083_at	1.658	FB:FBgn0034390 /sym=CG15093 /name= /prod=3-hydroxyisobutyrate dehydrogenase-like /func=enzyme
112	146620_s_at	1.655	FB:FBgn0032985 /sym=CG12628 /name= /prod=microsomal glutathione S-transferase-like /func=enzyme
113	149756_at	1.646	FB:FBgn0038021 /sym=CG4181 /name= /prod= /func=
114	142143_at	1.64	FB:FBgn0016078 /sym=wun /name=wunen /prod=phosphatidate phosphatase, type 2 /func=phosphatidate phosphatase
115	143602_at	1.634	FB:FBgn0010357 /sym=betaTry /name=betaTrypsin /prod=trypsin /func=trypsin
116	147124_at	1.626	FB:FBgn0033786 /sym=CG3884 /name= /prod= /func=
117	153443_at	1.62	FB:FBgn0038925 /sym=CG6022 /name= /prod=holocytochrome-C synthase /func=enzyme
118	151421_at	1.618	FB:FBgn0040958 /sym=CG13395 /name= /prod= /func=
119	153481_at	1.616	FB:FBgn0030672 /sym=CG9281 /name= /prod=ATP-binding cassette transporter /func=enzyme
120	145307_at	1.606	FB:FBgn0030929 /sym=CG15043 /name= /prod= /func=
121	149731_at	1.597	FB:FBgn0037989 /sym=CG14741 /name= /prod=P-type ATPase /func=transporter
122	150432_at	1.592	FB:FBgn0039062 /sym=CG17894 /name= /prod= /func=
123	149838_at	1.591	FB:FBgn0038147 /sym=CG14375 /name= /prod= /func=
124	152676_at	1.587	FB:FBgn0035391 /sym=CG2159 /name= /prod= /func=diacylglycerol kinase
125	141588_at	1.582	FB:FBgn0003742 /sym=tra2 /name=transformer 2 /prod= /func=RNA binding
126	150659_at	1.567	FB:FBgn0039428 /sym=CG14237 /name= /prod= /func=
127	153069_at	1.566	FB:FBgn0034394 /sym=CG15096 /name= /prod=sodium/phosphate cotransporter /func=transporter
128	150924_at	1.564	FB:FBgn0039818 /sym=CG11318 /name= /prod= /func=G protein linked receptor
129	147405_at	1.562	FB:FBgn0034288 /sym=CG5084 /name= /prod= /func=
130	153501_at	1.561	FB:FBgn0032053 /sym=CG13098 /name= /prod= /func=
131	151387_at	1.555	FB:FBgn0040918 /sym=CG15898 /name= /prod= /func=
132	148424_at	1.543	FB:FBgn0035929 /sym=CG13311 /name= /prod= /func=
133	147187_at	1.542	FB:FBgn0033903 /sym=CG8323 /name= /prod=oxaloacetate/sulfate carrier protein /func=carrier type transporter
134	152308_at	1.538	FB:FBgn0034638 /sym=CG10433 /name= /prod= /func=
135	144596_at	1.532	FB:FBgn0029869 /sym=CG3861 /name= /prod= /func=enzyme
136	141310_at	1.531	FB:FBgn0039760 /sym=CG9682 /name= /prod= /func=
137	155026_at	1.526	FB:FBgn0030612 /sym=CG5599 /name= /prod= /func=enzyme
138	150687_at	1.525	FB:FBgn0039457 /sym=CG6396 /name= /prod= /func=
139	152608_at	1.525	FB:FBgn0038972 /sym=CG7054 /name= /prod=phosphatidylethanolamine binding protein-like /func=ligand binding or carrier
140	146053_at	1.517	FB:FBgn0032075 /sym=CG9496 /name=Tetraspanin 29Fb /prod=transmembrane 4 superfamily member 6-like /func=
141	155046_at	1.515	FB:FBgn0029906 /sym=CG4542 /name= /prod= /func=enzyme
142	145074_at	1.514	FB:FBgn0030575 /sym=CG5321 /name= /prod= /func=
143	148843_at	1.514	FB:FBgn0036563 /sym=CG13075 /name= /prod= /func=
144	150049_at	1.504	FB:FBgn0038480 /sym=CG5233 /name= /prod=chymotrypsin-like serine protease /func=endopeptidase
145	151422_at	1.504	FB:FBgn0040959 /sym=CG17814 /name= /prod= /func=
146	150580_at	1.502	FB:FBgn0039317 /sym=CG10634 /name= /prod= /func=

Normal food vs. 00.5 mM Cd, genes downregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold downregulation	gene
1	152830_at	3.846	FB:FBgn0035154 /sym=CG3344 /name= /prod= /func=peptidase
2	150192_at	2.11	FB:FBgn0038701 /sym=CG18493 /name= /prod= /func=
3	141290_at	2.066	FB:FBgn0034494 /sym=CG10444 /name= /prod=sodium-dependent multivitamin transporter-like /func=transporter
4	144913_at	1.76	FB:FBgn0030366 /sym=CG1490 /name= /prod=ubiquitin thiolesterase /func=endopeptidase
5	153085_at	1.709	FB:FBgn0014019 /sym=Rh5 /name=Rhodopsin 5 /prod=rhodopsin 5 /func=light-sensitive visual pigment
6	147494_at	1.678	FB:FBgn0034440 /sym=CG10073 /name= /prod= /func=
7	142429_at	1.658	FB:FBgn0036861 /sym=CG14089 /name= /prod= /func=
8	143635_at	1.546	FB:FBgn0010808 /sym=l(3)03670 /name= /prod= /func=
9	146181_at	1.531	FB:FBgn0032282 /sym=CG7299 /name= /prod= /func=
10	151565_at	1.511	BDGP:GH2739.3prime-hit /ESTpos=maps in FB:FBgn0033790 /sym=CG3915 /name= /prod=/func=protein tyrosine kinase
11	148381_r_at	1.499	FB:FBgn0035858 /sym=CG13674 /name= /prod= /func=

Normal food vs. 0.5mM Cu, genes downregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold downregulation	gene
1	149489_at	3.8911	FB:FBgn0037575 /sym=CG7459 /name= /prod=copper transporter-like /func=transporter
2	153675_at	3.0675	FB:FBgn0003964 /sym=usp /name=ultraspiracle /prod=nuclear receptor NR2B4 /func=ecdysteroid hormone receptor
3	147494_at	2.3697	FB:FBgn0034440 /sym=CG10073 /name= /prod= /func=
4	148218_at	2.1505	FB:FBgn0035603 /sym=CG10635 /name= /prod= /func=
5	143868_at	2.1008	FB:FBgn0016075 /sym=vkg /name=viking /prod=collagen IV alpha2 chain /func=structural protein
6	142662_at	2.0704	FB:FBgn0038613 /sym=CG7678 /name= /prod=vacuolar ATPase, subunit-like /func=transporter
7	151641_at	1.9531	BDGP:LD1473.3prime-hit /ESTpos=maps in FB:FBgn0032890 /sym=CG9332 /name= /prod=glycerate dehydrogenase-like enzyme
8	152301_at	1.9493	FB:FBgn0033477 /sym=CG12918 /name= /prod= /func=
9	153363_at	1.9417	FB:FBgn0038976 /sym=CG7048 /name= /prod= /func=
10	149275_at	1.9011	FB:FBgn0037239 /sym=CG11739 /name= /prod= /func= /map=82B2-82B2 /transc=CT36785 /len=1274 /GB:AE003606
11	148041_at	1.8939	FB:FBgn0035335 /sym=CG1320 /name= /prod=mitochondrial ribosomal protein, L23-like /func=structural protein of ribosome
12	146181_at	1.8182	FB:FBgn0032282 /sym=CG7299 /name= /prod= /func= /map=32A1-32A1 /transc=CT22515 /len=534 /GB:AE003629
13	147104_at	1.8051	FB:FBgn0033754 /sym=CG8816 /name= /prod= /func= /map=49B2-49B2 /transc=CT25376 /len=590 /GB:AE003821
14	143945_at	1.7921	FB:FBgn0020907 /sym=Scp2 /name=Sarcoplasmic Ca-binding protein 2 /prod=sarcoplasmic Ca-binding protein 2 /func=Ca binding
15	154922_at	1.7857	FB:FBgn0039690 /sym=CG1969 /name= /prod= /func=glucosamine-phosphate N-acetyltransferase
16	152351_at	1.7762	FB:FBgn0035950 /sym=CG5288 /name= /prod= /func=enzyme
17	153449_at	1.773	FB:FBgn0032198 /sym=CG4912 /name= /prod= /func=translation factor
18	142167_at	1.7637	FB:FBgn0031497 /sym=CG17259 /name= /prod=serine--tRNA ligase-like /func=enzyme
19	153574_at	1.7544	FB:FBgn0027095 /sym=ARP-like /name= /prod=ARP-like /func=
20	143007_at	1.7513	FB:FBgn0001961 /sym=Sop2 /name=Suppressor of profilin 2/prod=actin related complex p41 subunit/func=ligand binding or carrier
21	152717_at	1.7422	FB:FBgn0023507 /sym=EG:87B1.3 /name= /prod=actin binding protein /func=actin binding
22	152469_at	1.7391	FB:FBgn0034118 /sym=CG6251 /name= /prod=nuclear pore complex glycoprotein /func=motor
23	151789_at	1.7301	FB:FBgn0035298 /sym=CG1140 /name= /prod= /func=glycoprotein-fucosylgalactoside alpha-N-acetylgalactosaminyltransferase
24	141539_at	1.7271	FB:FBgn0032134 /sym=CG3864 /name= /prod= /func=
25	153695_at	1.7241	FB:FBgn0030878 /sym=CG6769 /name= /prod= /func=transcription factor
26	151477_at	1.7182	BDGP:GH1453.3prime-hit /ESTpos=maps in FB:FBgn0031737/sym=CG11142/name=/prod=peritrophin-like /func=structural protein
27	152779_at	1.7094	FB:FBgn0038136 /sym=CG8774 /name= /prod=glutamyl aminopeptidase /func=peptidase
28	145815_at	1.7036	FB:FBgn0031692 /sym=CG6514 /name= /prod= /func=ligand binding or carrier
29	141232_at	1.7036	FB:FBgn0038326 /sym=CG5044 /name= /prod= /func=enoyl-CoA hydratase
30	152232_at	1.7007	FB:FBgn0033235 /sym=CG8728 /name= /prod=mitochondrial processing peptidase, alpha-chain /func=endopeptidase
31	143216_at	1.6892	FB:FBgn0001970 /sym=l(2)35Aa /name= /prod=/func=polypeptide N-acetylgalactosaminyltransferase
32	154207_at	1.6779	FB:FBgn0033734 /sym=CG8520 /name= /prod= /func=enzyme
33	152009_at	1.675	FB:FBgn0030692 /sym=CG8470 /name= /prod= /func=
34	154595_at	1.675	FB:FBgn0035798 /sym=CG7526 /name= /prod=fibrillin 2-like /func=cell adhesion
35	150980_at	1.6722	FB:FBgn0039909 /sym=CG1970 /name= /prod=NADH-ubiquinone oxidoreductase /func=enzyme
36	144595_at	1.6639	FB:FBgn0029868 /sym=CG3446 /name= /prod= /func=
37	153311_at	1.6611	FB:FBgn0001220 /sym=Hsc70-5 /name=Heat shock protein cognate 5 /prod=hsp cognate /func=mitochondrial chaperone
38	154671_at	1.6584	FB:FBgn0039094 /sym=CG10184 /name= /prod=threonine aldolase-like /func=enzyme
39	146084_at	1.6529	FB:FBgn0032114 /sym=CG3752 /name= /prod=aldehyde dehydrogenase /func=enzyme
40	153779_at	1.6393	FB:FBgn0000368 /sym=crb /name=crumbs /prod= /func=cell adhesion
41	152208_at	1.6367	FB:FBgn0010470 /sym=Fkbp13 /name= /prod=FK506 binding protein /func=FK56 binding
42	148367_at	1.6287	FB:FBgn0035827 /sym=CG8268 /name= /prod=signal recognition particle /func=RNA binding
43	143928_at	1.626	FB:FBgn0020443 /sym=Elf/name=Ef1alpha-like factor/prod=peptide chain release f.GTP-binding subunit;cytosolic transl. Release
44	146998_at	1.6207	FB:FBgn0033600 /sym=CG9077 /name= /prod=cuticle protein /func=structural protein
45	142788_at	1.6103	FB:FBgn0031043 /sym=CG14222 /name= /prod= /func=
46	153200_at	1.6103	FB:FBgn0031172 /sym=CG1704 /name= /prod=myosin binding protein-like /func=structural protein
47	143635_at	1.5949	FB:FBgn0010808 /sym=l(3)03670 /name= /prod= /func=
48	152661_at	1.5924	FB:FBgn0030749 /sym=Anxb11 /name=Annexin B11 /prod=annexin /func=calcium-dependent phospholipid binding

49	150049_at	1.5898	FB:FBgn0038480 /sym=CG5233 /name= /prod=chymotrypsin-like serine protease /func=endopeptidase
50	154053_at	1.5848	FB:FBgn0020653 /sym=Gr /name=Glutathione reductase /prod=/func=glutathione reductase (NADPH)
51	154049_at	1.5798	FB:FBgn0036444 /sym=CG9370 /name= /prod= /func=
52	154620_at	1.5773	FB:FBgn0032340 /sym=CG6181 /name= /prod= /func=motor
53	144156_at	1.5748	FB:FBgn0026753 /sym=Vha13 /name=Vacuolar H<up>+</up> ATPase/prod=H-transporting ATPase.G subunit /func=H-transport
54	148735_at	1.5649	FB:FBgn0036393 /sym=CG17362 /name= /prod= /func=
55	152452_at	1.5552	FB:FBgn0020415 /sym=Idgf2 /name=Imaginal Disc Growth Factor 2 /prod=/func=imaginal disc growth factor 2
56	141704_at	1.5456	FB:FBgn0032456 /sym=CG6214 /name= /prod=ATP-binding cassette transporter; multidrug resistance protein-like/func=ion channel
57	152126_at	1.5361	FB:FBgn0027571 /sym=BcDNA:GH07626 /name= /prod= /func=enzyme
58	154957_at	1.5361	FB:FBgn0035947 /sym=CG5064 /name= /prod=signal recognition particle protein 68-like /func=protein biosynthesis
59	154675_at	1.5337	FB:FBgn0029978 /sym=CG1515 /name= /prod= /func=
60	146986_at	1.5337	FB:FBgn0033588 /sym=CG13228 /name= /prod= /func=
61	141204_at	1.5337	FB:FBgn0032643 /sym=CG6453 /name= /prod= /func=receptor
62	153946_at	1.5314	FB:FBgn0037358 /sym=CG2185 /name= /prod=protein phosphatase-like /func=protein phosphatase
63	141746_at	1.5291	FB:FBgn0038519 /sym=CG5826 /name= /prod= /func=enzyme
64	148426_at	1.5267	FB:FBgn0035931 /sym=CG13312 /name= /prod= /func=
65	155109_at	1.5244	FB:FBgn0025617 /sym=EG:34F3.8 /name= /prod=vesicle transport protein /func=vesicle transport protein
66	148588_at	1.5152	FB:FBgn0036181 /sym=CG18331 /name= /prod= /func=
67	145955_at	1.5129	FB:FBgn0031912 /sym=CG5261 /name= /prod= /func=enzyme
68	152911_at	1.5129	FB:FBgn0017619 /sym=Fad /name=Fatty acid desaturase /prod=stearoyl-CoA desaturase /func=stearoyl-CoA desaturase
69	148668_at	1.506	FB:FBgn0036300 /sym=CG10688 /name= /prod=phosphomannomutase-like /func=enzyme
70	155162_at	1.506	FB:FBgn0034395 /sym=CG15081 /name= /prod= /func=
71	148837_at	1.4993	FB:FBgn0036551 /sym=CG17029 /name= /prod= /func=

Normal food vs. 5mM Zn, genes downregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold downregulation	gene
1	148183_at	3.571	FB:FBgn0035552 /sym=CG11350 /name= /prod= /func= /map=64B12-64B12 /transc=CT31662 /len=1101 /GB:AE003481
2	150052_at	3.268	FB:FBgn0038483 /sym=CG5240 /name= /prod= /func=endopeptidase /map=89F1-89F2 /transc=CT16729 /len=1629 /GB:AE00371
3	150050_at	2.841	FB:FBgn0038481 /sym=CG17475 /name= /prod=chymotrypsin-like serine protease /func=endopeptidase
4	144941_at	2.825	FB:FBgn0030394 /sym=CG2560 /name= /prod=cuticle protein-like /func=structural protein
5	142136_at	2.809	FB:FBgn0031737 /sym=CG11142 /name= /prod=peritrophin-like /func=structural protein
6	153224_at	2.717	FB:FBgn0033128 /sym=CG12142 /name=Tetraspanin 42Eg /prod=tetraspanin /func=
7	147339_at	2.551	FB:FBgn0034166 /sym=CG6472 /name= /prod=lipase /func=enzyme
8	150251_at	2.545	FB:FBgn0038783 /sym=CG4367 /name= /prod= /func= /map=92D9-92D9 /transc=CT14252 /len=642 /GB:AE003730
9	150206_at	2.513	FB:FBgn0038718 /sym=CG17752 /name= /prod=organic cation transporter-like /func=transporter
10	148426_at	2.451	FB:FBgn0035931 /sym=CG13312 /name= /prod= /func=
11	143401_at	2.439	FB:FBgn0003961 /sym=Uro /name=Urate oxidase /prod=urate oxidase /func=urate oxidase
12	144879_at	2.299	FB:FBgn0030305 /sym=CG1749 /name= /prod=molybdopterin synthase sulfurylase-like /func=enzyme
13	147857_at	2.268	FB:FBgn0035006 /sym=CG4563 /name= /prod=luciferase-like /func=enzyme
14	150045_at	2.257	FB:FBgn0038473 /sym=CG3983 /name= /prod= /func=
15	154405_at	2.247	FB:FBgn0029545 /sym=CG11642 /name= /prod= /func=
16	152301_at	2.217	FB:FBgn0033477 /sym=CG12918 /name= /prod= /func=
17	151477_at	2.203	BDGP:GH1453.3prime-hit /ESTpos=maps in FB:FBgn0031737 /sym=CG11142 /name= /prod=peritrophin-like /func=structural protei
18	146998_at	2.165	FB:FBgn0033600 /sym=CG9077 /name= /prod=cuticle protein /func=structural protein
19	150582_at	2.128	FB:FBgn0039319 /sym=CG13659 /name= /prod= /func=
20	150049_at	2.101	FB:FBgn0038480 /sym=CG5233 /name= /prod=chymotrypsin-like serine protease /func=endopeptidase
21	153750_at	2.075	FB:FBgn0031379 /sym=CG7289 /name= /prod= /func=
22	149172_at	2.045	FB:FBgn0037069 /sym=CG7658 /name= /prod=cuticle protein-like /func=structural protein
23	154671_at	2.024	FB:FBgn0039094 /sym=CG10184 /name= /prod=threonine aldolase-like /func=enzyme
24	150217_s_at	2.008	FB:FBgn0038731 /sym=CG11659 /name= /prod=luciferase-like /func=enzyme
25	145798_at	1.972	FB:FBgn0031656 /sym=CG8885 /name= /prod=cytochrome-c oxidase assembly protein-like /func=enzyme
26	145047_at	1.969	FB:FBgn0030541 /sym=CG11584 /name= /prod= /func=ligand binding or carrier
27	141360_at	1.927	FB:FBgn0036659 /sym=CG9701 /name= /prod=beta-glucosidase-like /func=ion channel
28	153946_at	1.923	FB:FBgn0037358 /sym=CG2185 /name= /prod=protein phosphatase-like /func=protein phosphatase
29	154661_at	1.912	FB:FBgn0032610 /sym=CG17937 /name= /prod=diacylglycerol O-acyltransferase-like /func=enzyme
30	141546_at	1.908	FB:FBgn0037250 /sym=CG1074 /name= /prod= /func=
31	147494_at	1.901	FB:FBgn0034440 /sym=CG10073 /name= /prod= /func=
32	151805_at	1.89	FB:FBgn0016718 /sym=Reg-3 /name= /prod= /func=enzyme
33	153449_at	1.88	FB:FBgn0032198 /sym=CG4912 /name= /prod= /func=translation factor
34	152083_at	1.873	FB:FBgn0034390 /sym=CG15093 /name= /prod=3-hydroxyisobutyrate dehydrogenase-like /func=enzyme
35	150424_at	1.869	FB:FBgn0039052 /sym=CG6733 /name= /prod=aminoacylase-like (inactive) /func=peptidase
36	153279_at	1.859	FB:FBgn0027912 /sym=BcDNA:GM12291 /name= /prod= /func=
37	153033_at	1.852	FB:FBgn0038610 /sym=CG7675 /name= /prod= /func=enzyme
38	153752_at	1.842	FB:FBgn0024558 /sym=Dph5 /name=Diphthamide methyltransferase /prod=diphthine synthase /func=diphthine synthase
39	143547_at	1.802	FB:FBgn0005585 /sym=Crc /name=Calreticulin /prod=calreticulin /func=calcium binding
40	150239_at	1.799	FB:FBgn0038764 /sym=CG4845 /name= /prod= /func=actin binding
41	153200_at	1.799	FB:FBgn0031172 /sym=CG1704 /name= /prod=myosin binding protein-like /func=structural protein
42	154500_at	1.783	FB:FBgn0003470 /sym=alpha-Spec /name=alpha Spectrin /prod=alpha-spectrin /func=actin cross-linking
43	154595_at	1.776	FB:FBgn0035798 /sym=CG7526 /name= /prod=fibrillin 2-like /func=cell adhesion
44	151840_at	1.77	FB:FBgn0027615 /sym=BcDNA:GH02220 /name= /prod=cytochrome c oxidase assembly protein-like /func=enzyme
45	146692_at	1.751	FB:FBgn0033103 /sym=CG15235 /name= /prod= /func=signal transduction
46	141420_at	1.73	FB:FBgn0025620 /sym=EG:34F3.5 /name= /prod= /func=
47	148218_at	1.718	FB:FBgn0035603 /sym=CG10635 /name= /prod= /func=
48	143945_at	1.709	FB:FBgn0020907 /sym=Scp2 /name=Sarcoplasmic Ca-binding protein 2 /prod=sarcoplasmic Ca-binding protein 2 /func=Ca binding
49	155126_at	1.706	FB:FBgn0036312 /sym=CG17667 /name= /prod= /func=cell adhesion
50	152373_at	1.704	FB:FBgn0037701 /sym=CG9373 /name= /prod= /func=RNA binding
51	143912_at	1.704	FB:FBgn0020236 /sym=ATPCL /name=ATP citrate lyase /prod=ATP-citrate (pro-S)-lyase /func=ATP-citrate (pro-S)-lyase
52	141350_at	1.686	FB:FBgn0031657 /sym=CG3756 /name= /prod=RNA polymerase I, 40kD subunit /func=enzyme
53	144088_at	1.681	FB:FBgn0025682 /sym=scf /name=supercoiling factor /prod=supercoiling factor /func=ligand binding or carrier

54	151287_at	1.678	FB:FBgn0040813 /sym=CG11051 /name= /prod= /func=
55	153790_at	1.675	FB:FBgn0029721 /sym=CG7010 /name= /prod= /func=enzyme
56	143628_at	1.675	FB:FBgn0010438 /sym=mtSSB /name=mitochondrial ssDNA-binding protein /prod=ssDNA binding protein /func=ssDNA binding
57	141603_at	1.661	FB:FBgn0033805 /sym=CG4062 /name= /prod= /func=
58	151733_at	1.658	BDGP:LP7553.3prime-hit /ESTpos=maps in FB:FBgn0032396 /sym=CG5304 name=/prod=/func=sodium/phosphate cotransporte
59	147104_at	1.637	FB:FBgn0033754 /sym=CG8816 /name= /prod= /func=
60	146986_at	1.634	FB:FBgn0033588 /sym=CG13228 /name= /prod= /func=
61	141311_at	1.618	FB:FBgn0036901 /sym=CG8756 /name= /prod=low-density lipoprotein-receptor-like /func=receptor
62	141400_at	1.613	FB:FBgn0037894 /sym=CG5252 /name= /prod=Ran binding protein 9 /func=RAN protein binding
63	153574_at	1.605	FB:FBgn0027095 /sym=ARP-like /name= /prod=ARP-like /func=
64	153498_at	1.603	FB:FBgn0027897 /sym=BcDNA:LD03471 /name= /prod= /func=
65	144971_at	1.597	FB:FBgn0030433 /sym=CG4647 /name= /prod= /func=
66	145732_at	1.597	FB:FBgn0031562 /sym=CG3604 /name= /prod=trypsin inhibitor-like /func=enzyme inhibitor
67	145611_at	1.59	FB:FBgn0031378 /sym=CG15362 /name= /prod=PKC inhibitor /func=signal transduction
68	141387_at	1.58	FB:FBgn0035473 /sym=CG14981 /name=maggie /prod=outer mitochondrial translocase /func=
69	154683_at	1.55	FB:FBgn0037608 /sym=CG8039 /name= /prod=ribosomal protein L subunit-like /func=structural protein of ribosome
70	150031_at	1.541	FB:FBgn0038450 /sym=CG17560 /name= /prod= /func=
71	144913_at	1.534	FB:FBgn0030366 /sym=CG1490 /name= /prod=ubiquitin thiolesterase /func=endopeptidase
72	143632_at	1.529	FB:FBgn0010747 /sym=Srp54k /name=Signal recognition particle protein 54k /prod=SRP 54kD /func=protein signal sequence bindir
73	144432_at	1.524	FB:FBgn0029652 /sym=CG14265 /name= /prod= /func=
74	153391_at	1.517	FB:FBgn0028544 /sym=BG:DS00180.3 /name= /prod= /func=
75	147793_at	1.517	FB:FBgn0034885 /sym=CG4019 /name= /prod=water transporter-like /func=transporter
76	152081_at	1.515	FB:FBgn0036760 /sym=CG5567 /name= /prod=4-nitrophenylphosphatase-like /func=enzyme
77	153238_at	1.513	FB:FBgn0038138 /sym=CG8775 /name= /prod=glutamyl aminopeptidase /func=peptidase
78	141617_at	1.513	FB:FBgn0010339 /sym=128up /name=upstream of RpIII128 /prod=GTP binding protein /func=GTP binding
79	153950_at	1.511	FB:FBgn0000463 /sym=Dl /name=Delta /prod= /func=Notch receptor ligand
80	144168_at	1.508	FB:FBgn0026879 /sym=EG:115C2.12 /name= /prod= /func=
81	147265_at	1.504	FB:FBgn0034045 /sym=CG8249 /name= /prod=glucose transporter-like /func=transporter
82	144119_at	1.502	FB:FBgn0026089 /sym=EG:63B12.11 /name= /prod= /func=

Normal food vs. 0.5 mM BCS, genes downregulated more than 1.5 fold, p-value ≤ 0.05

	Probe set ID	fold down-regulation	gene
1	147494_at	76.923	FB:FBgn0034440 /sym=CG10073 /name= /prod= /func=
2	150257_at	28.571	FB:FBgn0038790 /sym=CG5097 /name= /prod=metallothionein /func=ligand binding or carrier
3	143276_at	26.316	FB:FBgn0002868 /sym=MtnA /name=Metallothionein A /prod=metallothionein A /func=Cu/Cd binding
4	150702_at	13.514	FB:FBgn0039474 /sym=CG6283 /name= /prod=triacylglycerol lipase /func=enzyme
5	142461_at	10.101	FB:FBgn0034901 /sym=CG11300 /name= /prod= /func=
6	147487_r_at	9.009	FB:FBgn0034428 /sym=CG18606 /name= /prod= /func=
7	147243_at	7.4627	FB:FBgn0034011 /sym=CG8160 /name= /prod= /func=
8	150701_at	6.8027	FB:FBgn0039473 /sym=CG17191 /name= /prod=lipase-like /func=enzyme
9	147486_i_at	5.3763	FB:FBgn0034428 /sym=CG18606 /name= /prod= /func=
10	147484_s_at	5.2632	FB:FBgn0034426 /sym=CG10476 /name= /prod= /func=enzyme
11	152900_at	4.3478	FB:FBgn0025454 /sym=Cyp6g1 /name= /prod=cytochrome P450, CYP6G1 /func=cytochrome P45 ; EC:1.14.14.1
12	153583_at	4.2373	FB:FBgn0001224 /sym=Hsp23 /name=Heat shock protein 23 /prod=heat shock protein 23 kD /func=heat shock protein
13	149172_at	3.4247	FB:FBgn0037069 /sym=CG7658 /name= /prod=cuticle protein-like /func=structural protein
14	154717_at	3.413	FB:FBgn0033840 /sym=CG4840 /name= /prod= /func=motor
15	143295_at	2.9499	FB:FBgn0003046 /sym=Pcp /name=Pupal cuticle protein /prod= /func=structural protein of pupal cuticle (Drosophila)
16	148735_at	2.924	FB:FBgn0036393 /sym=CG17362 /name= /prod= /func=
17	147514_at	2.8818	FB:FBgn0034468 /sym=CG11797 /name= /prod= /func=
18	154922_at	2.8011	FB:FBgn0039690 /sym=CG1969 /name= /prod= /func=glucosamine-phosphate N-acetyltransferase ; EC:2.3.1.4
19	153365_at	2.7778	FB:FBgn0032773 /sym=CG15825 /name= /prod= /func=
20	147336_at	2.7174	FB:FBgn0034162 /sym=CG6426 /name= /prod=calcium binding protein-like /func=ligand binding or carrier
21	150837_at	2.71	FB:FBgn0039685 /sym=CG7592 /name= /prod=odorant binding-like protein /func=ligand binding or carrier
22	152173_at	2.6596	FB:FBgn0010406 /sym=RNaseX25 /name=Ribonuclease X25 /prod=ribonuclease-like /func=ribonuclease
23	150090_at	2.6455	FB:FBgn0038530 /sym=CG7629 /name= /prod= /func=defense/immunity protein
24	147483_i_at	2.6247	FB:FBgn0034426 /sym=CG10476 /name= /prod= /func=enzyme
25	153950_at	2.6042	FB:FBgn0000463 /sym=Dl /name=Delta /prod= /func=Notch receptor ligand
26	150273_at	2.5316	FB:FBgn0038819 /sym=CG5494 /name= /prod=cuticle protein /func=structural protein
27	145728_at	2.4752	FB:FBgn0031558 /sym=CG16704 /name= /prod= /func=
28	143955_at	2.457	FB:FBgn0022341 /sym=CG17467 /name= /prod= /func=
29	147450_at	2.4331	FB:FBgn0034364 /sym=CG5493 /name= /prod= /func=
30	154674_at	2.4331	FB:FBgn0039606 /sym=CG1448 /name= /prod= /func=
31	153767_at	2.4155	FB:FBgn0038249 /sym=CG7832 /name= /prod= /func=
32	145511_at	2.4096	FB:FBgn0031221 /sym=CG3164 /name= /prod=ATP-binding cassette transporter /func=enzyme
33	151315_at	2.3981	FB:FBgn0040842 /sym=CG15212 /name= /prod= /func=
34	153977_at	2.3474	FB:FBgn0022770 /sym=Peritrophin-A /name=Peritrophin A /prod=peritrophin A /func=chitin binding
35	153374_at	2.3419	FB:FBgn0004646 /sym=ogre /name=optic ganglion reduced /prod=innexin /func=ion channel
36	153675_at	2.3095	FB:FBgn0003964 /sym=usp /name=ultraspiracle /prod=nuclear receptor NR2B4 /func=ecdysteroid hormone receptor
37	143682_at	2.3041	FB:FBgn0011722 /sym=Tig /name=Tiggrin /prod= /func=motor
38	145730_at	2.2989	FB:FBgn0031560 /sym=CG16713 /name= /prod= /func=
39	149850_at	2.2883	FB:FBgn0038161 /sym=CG9269 /name= /prod= /func=
40	153679_at	2.2727	FB:FBgn0022342 /sym=CG4844 /name= /prod= /func=ligand binding or carrier
41	146998_at	2.2573	FB:FBgn0033600 /sym=CG9077 /name= /prod=cuticle protein /func=structural protein
42	147891_at	2.2573	FB:FBgn0035062 /sym=CG16914 /name= /prod=larval cuticle protein-like /func=structural protein
43	147740_at	2.2371	FB:FBgn0034819 /sym=CG9877 /name= /prod= /func=
44	141360_at	2.2321	FB:FBgn0036659 /sym=CG9701 /name= /prod=beta-glucosidase-like /func=ion channel
45	143623_at	2.2272	FB:FBgn0010424 /sym=TpnC73F /name=Troponin C at 73F /prod=troponin C /func=calcium binding
46	154791_at	2.1978	FB:FBgn0031037 /sym=CG14207 /name= /prod=heat shock protein HSP20-like /func=chaperone
47	152160_at	2.1882	FB:FBgn0015239 /sym=Hr78 /name=Hormone-receptor-like in 78 /prod=nuclear receptor NR2D1 /func=ligand-dependent nuclear rece
48	151467_at	2.1834	BDGP:GH159.3'-hit/EST /sym=emb/name=embargoed/prod=exportin/func=nuclear export signal receptor
49	152782_at	2.1786	FB:FBgn0037721 /sym=CG9427 /name= /prod= /func=
50	149607_at	2.1739	FB:FBgn0037779 /sym=CG12811 /name= /prod= /func=
51	145050_at	2.1598	FB:FBgn0030544 /sym=CG13403 /name= /prod= /func=
52	143945_at	2.1552	FB:FBgn0020907 /sym=Scp2 /name=Sarcoplasmic Ca-binding protein 2 /prod=sarcoplasmic Ca-binding protein 2 /func=calcium bindir
53	154351_at	2.1277	FB:FBgn0020369 /sym=Pros45 /name=UAS constr.of Cheng/19S proteasome regul. particle, triple-A pr, subunit S8; proteasome ATPa

54	154405_at	2.1231	FB:FBgn0029545 /sym=CG11642 /name= /prod= /func=
55	151477_at	2.1186	BDGP:GH1453.3prime-hit /ESTpos=maps in FB:FBgn0031737 /sym=CG11142 /name= /prod=peritrophin-like /func=structural protein
56	148873_at	2.1097	FB:FBgn0036600 /sym=CG13043 /name= /prod= /func=
57	145265_at	2.1008	FB:FBgn0030876 /sym=CG6762 /name= /prod= /func=
58	153527_at	2.0921	FB:FBgn0030309 /sym=CG1572 /name= /prod= /func=
59	153111_at	2.0877	FB:FBgn0037848 /sym=CG4591 /name= /prod= /func=transmembrane receptor
60	155056_at	2.0833	FB:FBgn0023174 /sym=Prosbeta2 /name=Proteasome beta2 subunit /prod=20S proteasome, beta2/func=multicatalytic endopeptidase
61	141420_at	2.0747	FB:FBgn0025620 /sym=EG:34F3.5 /name= /prod= /func=
62	152591_at	2.0704	FB:FBgn0034200 /sym=CG11395 /name= /prod= /func=structural protein
63	151921_at	2.0492	FB:FBgn0002968 /sym=Nrg /name=Neuroglian /prod=neuroglian /func=cell adhesion
64	148295_at	2.045	FB:FBgn0035726 /sym=CG9953 /name= /prod=lysosomal pro-X carboxypeptidase /func=lysosomal pro-X carboxypeptidase
65	144143_at	2.0408	FB:FBgn0026415 /sym=Idgf4 /name=Imaginal Disc Growth Factor 4 /prod=/func=imaginal disc growth factor 4
66	152688_at	2.0325	FB:FBgn0036673 /sym=CG11915 /name= /prod= /func=ligand binding or carrier
67	142822_at	2.0325	FB:FBgn0032727 /sym=CG10623 /name= /prod= /func=
68	143239_at	2.0202	FB:FBgn0002565 /sym=Lsp2 /name=Larval serum protein 2 /prod=larval serum protein 2 /func=larval serum protein
69	151951_at	2	FB:FBgn0037466 /sym=CG1965 /name= /prod= /func=G protein linked receptor
70	154581_at	2	FB:FBgn0036302 /sym=CG10632 /name= /prod= /func=enzyme
71	145414_at	1.996	FB:FBgn0031089 /sym=CG9572 /name= /prod= /func=
72	145612_at	1.992	FB:FBgn0031381 /sym=CG7291 /name= /prod= /func=signal transduction
73	144182_at	1.992	FB:FBgn0027497 /sym=BcDNA:LD28657 /name= /prod=protein serine/threonine kinase-like /func=protein kinase
74	143132_at	1.992	FB:FBgn0000497 /sym=ds /name=dachsous /prod=cadherin /func=Ca<up>2+</up>-dependent cell adhesion
75	152368_at	1.9881	FB:FBgn0038465 /sym=CG8913 /name= /prod= /func=enzyme
76	148781_at	1.9802	FB:FBgn0036467 /sym=CG12310 /name= /prod= /func=
77	148410_at	1.9802	FB:FBgn0035904 /sym=CG6776 /name= /prod=glutathione transferase /func=glutathione transferase
78	151775_at	1.9802	FB:FBgn0037357 /sym=sec23 /name= /prod= /func=GTPase activator
79	141762_at	1.9802	FB:FBgn0013770 /sym=Cp1 /name=Cysteine proteinase-1 /prod=cathepsin L /func=cathepsin L ; EC:3.4.22.15
80	148536_at	1.9763	FB:FBgn0036107 /sym=CG7949 /name= /prod= /func=
81	153252_at	1.9763	FB:FBgn0029867 /sym=CG3847 /name= /prod= /func=nucleic acid binding
82	154085_at	1.9724	FB:FBgn0000566 /sym=Eip55E /name= /prod=cystathionine-gamma-lyase /func=cystathionine-gamma-lyase ; EC:4.4.1.1
83	148181_at	1.9685	FB:FBgn0035550 /sym=CG11349 /name= /prod= /func=structural protein
84	153539_at	1.9685	FB:FBgn0026077 /sym=Gasp /name= /prod=peritrophin-like /func=chitin binding
85	147551_at	1.9493	FB:FBgn0034516 /sym=CG13429 /name= /prod=gram-negative binding protein /func=defense/immunity protein
86	142982_at	1.938	FB:FBgn0015282 /sym=Pros26.4 /name=Proteasome 26S subunit 4 ATPase /prod=19S proteasome regul. particle, triple-A pr, subunit
87	149853_at	1.938	FB:FBgn0038166 /sym=CG9588 /name= /prod=19S proteasome regul. particle, non-ATPase protein p27,subunit S15/func=endopeptic
88	144338_at	1.9342	FB:FBgn0029517 /sym=CG13377 /name= /prod= /func=
89	154671_at	1.9305	FB:FBgn0039094 /sym=CG10184 /name= /prod=threonine aldolase-like /func=enzyme
90	147254_at	1.9231	FB:FBgn0034025 /sym=CG8182 /name= /prod=polypeptide N-acetylgalactosaminyltransferase /func=enzyme
91	148532_at	1.9231	FB:FBgn0036100 /sym=CG6463 /name= /prod= /func=NADH dehydrogenase
92	142413_at	1.9157	FB:FBgn0039800 /sym=CG11314 /name= /prod= /func=
93	154446_at	1.912	FB:FBgn0037240 /sym=CG1084 /name= /prod=neural cell adhesion protein-like /func=cell adhesion
94	152457_at	1.9084	FB:FBgn0035985 /sym=CG3672 /name= /prod= /func=structural protein
95	141527_at	1.9011	FB:FBgn0023479 /sym=Tequila /name= /prod=serine endopeptidase /func=serine-type endopeptidase
96	153122_at	1.8975	FB:FBgn0000052 /sym=ade2 /name=adenosine 2 /prod= /func=phosphoribosylformylglycinamidine synthase ; EC:6.3.5.3
97	152504_at	1.8939	FB:FBgn0014869 /sym=Pglym78 /name=Phosphoglyceromutase /prod=phosphoglycerate mutase /func=phosphoglycerate mutase
98	142395_at	1.8939	FB:FBgn0028542 /sym=BG:DS00180.8 /name= /prod= /func=cell adhesion
99	153467_at	1.8904	FB:FBgn0000283 /sym=Cen190 /name=Centrosomal protein 19kD /prod= /func=nucleic acid binding
100	154078_at	1.8904	FB:FBgn0014469 /sym=Cyp4e2 /name=Cytochrome P45-4e2 /prod=cytochrome P450, CYP4E2 /func=cytochrome P45 ; EC:1.14.14.1
101	153155_at	1.8868	FB:FBgn0024923 /sym=TER94 /name=UAS constr. of McKearin /prod=transitional endoplasmic reticulum adenosinetriphosphatase
102	146649_at	1.8797	FB:FBgn0033032 /sym=CG1298 /name= /prod= /func=
103	152607_at	1.8727	FB:FBgn0035769 /sym=CG8591 /name= /prod= /func=nucleic acid binding
104	153772_at	1.8692	FB:FBgn0010235 /sym=Klc /name=Kinesin light chain /prod=kinesin light chain /func=microtuble binding
105	155148_at	1.8657	FB:FBgn0034583 /sym=CG10527 /name= /prod= /func=
106	148785_at	1.8657	FB:FBgn0036471 /sym=CG13460 /name= /prod= /func=
107	143837_at	1.8657	FB:FBgn0015754 /sym=Lis1 /name=Lissencephaly-1 /prod=platelet-activating factor acetylhydrolase beta subunit-like /func=2-acetyl-1-alkylglycerophosphocholine esterase ; EC:3.1.1.47
108	142638_at	1.8622	FB:FBgn0014029 /sym=36771 /name=Septin-2 /prod=septin /func=GTP binding
109	152759_at	1.8553	FB:FBgn0032774 /sym=CG17549 /name= /prod= /func=
110	141697_at	1.8553	FB:FBgn0010225 /sym=Gel /name=Gelsolin /prod=gelsolin /func=actin binding
111	151019_at	1.8519	FB:FBgn0040532 /sym=CG8369 /name= /prod= /func=
112	154869_at	1.8484	FB:FBgn0035612 /sym=CG10625 /name= /prod= /func=

113	142853_at	1.8484	FB:FBgn0023175 /sym=Prosalpha7 /name=Proteasome alpha7 subunit/prod=20S proteasome, alpha7 s.u.; multicatalytic endopeptida
114	142656_at	1.8416	FB:FBgn0004556 /sym=Dbp73D /name=Dead box protein 73D /prod= /func=ATP dependent RNA helicase
115	151196_r_at	1.8349	FB:FBgn0040718 /sym=CG15353 /name= /prod= /func=
116	155069_at	1.8215	FB:FBgn0032693 /sym=Cyp310a1 /name= /prod=cytochrome P450, CYP310A1 /func=cytochrome P45
117	AFFX-Dros-GAPDH_3_at	1.8182	Drosophila gene for Gapdh2 (_5, _M, _3 represent transcript regions 5 prime, Middle, and 3 prime respectively)
118	154814_at	1.8116	FB:FBgn0000064 /sym=Ald /name=Aldolase /prod=fructose-bisphosphate aldolase /func=fructose-bisphosphate aldolase
119	153569_at	1.8116	FB:FBgn0032165 /sym=CG5879 /name= /prod=cuticle protein /func=structural protein
120	AFFX-Dros-ACTIN_3_at	1.8116	Drosophila gene for Actin 42A (_5, _M, _3 represent transcript regions 5 prime, Middle, and 3 prime respectively)
121	149757_at	1.8083	FB:FBgn0038022 /sym=CG4381 /name= /prod= /func=
122	151021_at	1.8083	FB:FBgn0040534 /sym=CG11985 /name= /prod= /func=
123	153857_at	1.8051	FB:FBgn0025741 /sym=plexA /name=plexin A /prod=semaphorin receptor /func=axon guidance receptor
124	152619_at	1.8018	FB:FBgn0034575 /sym=CG15652 /name= /prod= /func=
125	143427_at	1.7986	FB:FBgn0004117 /sym=Tm2 /name=Tropomyosin 2 /prod=tropomyosin /func=motor
126	145561_at	1.7953	FB:FBgn0031309 /sym=CG5041 /name= /prod=TFB4-like /func=general RNA polymerase II transcription factor
127	AFFX-Dros-GAPDH_M_at	1.7857	Drosophila gene for Gapdh2 (_5, _M, _3 represent transcript regions 5 prime, Middle, and 3 prime respectively)
128	145492_at	1.7825	FB:FBgn0031198 /sym=CG13238 /name= /prod= /func=
129	146351_i_at	1.773	FB:FBgn0032538 /sym=CG16885 /name= /prod= /func=
130	154661_at	1.7606	FB:FBgn0032610 /sym=CG17937 /name= /prod=diacylglycerol O-acyltransferase-like /func=enzyme
131	144220_at	1.7575	FB:FBgn0028510 /sym=BG:DS07851.3 /name= /prod= /func=
132	144882_at	1.7513	FB:FBgn0030310 /sym=CG11709 /name= /prod=peptidoglycan recognition protein-like /func=defense/immunity protein
133	152220_at	1.7513	FB:FBgn0034517 /sym=CG18066 /name= /prod= /func=
134	147436_at	1.7483	FB:FBgn0034337 /sym=CG17524 /name= /prod=glutathione transferase /func=enzyme
135	153835_at	1.7452	FB:FBgn0004893 /sym=bowl /name=brother of odd with entrails limited /prod= /func=RNA polymerase II transcription factor
136	152469_at	1.7452	FB:FBgn0034118 /sym=CG6251 /name= /prod=nuclear pore complex glycoprotein /func=motor
137	142969_at	1.7422	FB:FBgn0004629 /sym=Cys /name=Cystatin-like /prod=cystatin-like /func=cysteine protease inhibitor
138	149399_at	1.7422	FB:FBgn0037440 /sym=CG1041 /name= /prod= /func=enzyme
139	141260_at	1.7331	FB:FBgn0038738 /sym=CG4572 /name= /prod=vitellogenic carboxypeptidase /func=peptidase
140	148283_at	1.7301	FB:FBgn0035704 /sym=CG10144 /name= /prod= /func=
141	153413_at	1.7241	FB:FBgn0015801 /sym=Reg-5 /name=Rhythmically expressed gene 5 /prod= /func=
142	144026_at	1.7212	FB:FBgn0024841 /sym=Pcd /name=pterin-4a-carbinolamine dehydratase /prod=/func=4a-hydroxytetrahydrobiopterin dehydratase
143	152100_at	1.7123	FB:FBgn0028490 /sym=BcDNA:GH07269 /name= /prod= /func=DNA binding
144	153574_at	1.7036	FB:FBgn0027095 /sym=ARP-like /name= /prod=ARP-like /func=
145	151878_at	1.6978	FB:FBgn0028541 /sym=BG:DS00797.1 /name= /prod=/func=endosomal small-molecule carrier or transporter
146	147144_at	1.692	FB:FBgn0033826 /sym=CG4734 /name= /prod= /func=
147	144306_at	1.6892	FB:FBgn0028915 /sym=BG:DS01068.5 /name= /prod=serine endopeptidase /func=endopeptidase
148	141710_r_at	1.6892	FB:FBgn0003885 /sym=alphaTub84D /name=alphaTubulin84D /prod=alpha-tubulin /func=cytoskeletal structural protein
149	154843_at	1.6863	FB:FBgn0035850 /sym=CG7986 /name= /prod= /func=transcription factor
150	155031_at	1.6863	FB:FBgn0033346 /sym=CG11770 /name= /prod= /func=
151	141276_at	1.6807	FB:FBgn0022359 /sym=Sodh-2 /name=Sorbitol dehydrogenase-2 /prod=L-iditol 2-dehydrogenase /func=L-iditol 2-dehydrogenase
152	145732_at	1.6807	FB:FBgn0031562 /sym=CG3604 /name= /prod=trypsin inhibitor-like /func=enzyme inhibitor
153	153391_at	1.6779	FB:FBgn0028544 /sym=BG:DS00180.3 /name= /prod= /func=
154	142488_s_at	1.6779	FB:FBgn0037930 /sym=CG14715 /name= /prod= /func=chaperone
155	154209_at	1.675	FB:FBgn0037707 /sym=CG16788 /name= /prod= /func=RNA binding
156	154336_at	1.6722	FB:FBgn0000084 /sym=AnnX /name=Annexin X /prod=annexin X /func=calcium-dependent phospholipid binding
157	154804_at	1.6694	FB:FBgn0033352 /sym=CG8232 /name= /prod=PAB-dependent poly(A)-specific ribonuclease subunit /func=enzyme
158	153384_at	1.6639	FB:FBgn0031695 /sym=Cyp4ac3 /name= /prod=cytochrome P450, CYP4AC3 /func=cytochrome P45
159	154683_at	1.6611	FB:FBgn0037608 /sym=CG8039 /name= /prod=ribosomal protein L subunit-like /func=structural protein of ribosome
160	141515_at	1.6611	FB:FBgn0020930 /sym=Dgkepsilon /name=Diacyl glycerol kinase epsilon /prod=/func=diacylglycerol kinase
161	154977_at	1.6556	FB:FBgn0030306 /sym=CG1751 /name= /prod= /func=
162	154595_at	1.6502	FB:FBgn0035798 /sym=CG7526 /name= /prod=fibrillin 2-like /func=cell adhesion
163	151849_at	1.6502	FB:FBgn0010288 /sym=Uch /name=Ubiquitin carboxy-terminal hydrolase /prod=ubiquitinyl hydrolase 1 /func=ubiquitinyl hydrolase 1
164	154656_at	1.6474	FB:FBgn0038048 /sym=CG12276 /name= /prod= /func=
165	148218_at	1.6474	FB:FBgn0035603 /sym=CG10635 /name= /prod= /func=
166	152055_at	1.6447	FB:FBgn0035495 /sym=CG14989 /name= /prod= /func=
167	146935_at	1.6393	FB:FBgn0033506 /sym=CG3298 /name= /prod= /func=
168	142700_at	1.626	FB:FBgn0037732 /sym=CG9443 /name= /prod= /func=
169	145211_at	1.626	FB:FBgn0030788 /sym=CG4756 /name= /prod= /func=transcription factor
170	146986_at	1.6207	FB:FBgn0033588 /sym=CG13228 /name= /prod= /func=

171	147793_at	1.6181	FB:FBgn0034885 /sym=CG4019 /name= /prod=water transporter-like /func=transporter
172	152571_at	1.6103	FB:FBgn0038115 /sym=CG7966 /name= /prod=selenium-binding protein-like /func=ligand binding or carrier
173	144223_at	1.6103	FB:FBgn0028515 /sym=BG:DS07473.2 /name= /prod= /func=
174	148098_at	1.6103	FB:FBgn0035427 /sym=CG14959 /name= /prod= /func=
175	144244_at	1.6077	FB:FBgn0028685 /sym=Rpt4 /name= /prod=19S proteasome regulatory particle, triple-A protein, subunit S10b /func=proteasome ATP
176	154815_at	1.6077	FB:FBgn0020633 /sym=Mcm7 /name=Minichromosome maintenance 7 /prod=DNA replication licensing factor 7 /func=chromatin bindi
177	146503_at	1.6051	FB:FBgn0032806 /sym=CG10363 /name= /prod= /func=
178	152681_at	1.6	FB:FBgn0033624 /sym=CG12384 /name= /prod= /func=
179	143547_at	1.6	FB:FBgn0005585 /sym=Crc /name=Calreticulin /prod=calreticulin /func=calcium binding
180	143249_at	1.6	FB:FBgn0002622 /sym=RpS3 /name=Ribosomal protein S3 /prod=ribosomal protein S3 /func=DNA-(apurinic or apyrimidinic site) lyase
181	151195_i_at	1.5974	FB:FBgn0040718 /sym=CG15353 /name= /prod= /func=
182	153785_at	1.5949	FB:FBgn0034117 /sym=CG7997 /name= /prod= /func=enzyme
183	143184_at	1.5949	FB:FBgn0001149 /sym=GstD1 /name=Glutathione S transferase D1 /prod=glutathione transferase D1 /func=glutathione transferase
184	154374_at	1.5924	FB:FBgn0035631 /sym=CG5495 /name=Thioredoxin-like /prod=thioredoxin-like /func=thioredoxin
185	148668_at	1.5898	FB:FBgn0036300 /sym=CG10688 /name= /prod=phosphomannomutase-like /func=enzyme
186	150222_at	1.5898	FB:FBgn0038739 /sym=CG4686 /name= /prod= /func=
187	152255_at	1.5898	FB:FBgn0039464 /sym=CG6330 /name= /prod=uridine phosphorylase /func=enzyme
188	154080_at	1.5873	FB:FBgn0030093 /sym=CG7055 /name= /prod= /func=DNA binding
189	152613_at	1.5798	FB:FBgn0036337 /sym=CG11255 /name= /prod=adenosine kinase /func=enzyme
190	153292_at	1.5748	FB:FBgn0033055 /sym=CG7861 /name= /prod= /func=motor
191	155062_at	1.5748	FB:FBgn0015602 /sym=BEAF-32 /name=Boundary element-associated factor of 32kD /prod= /func=DNA binding
192	143264_at	1.5723	FB:FBgn0002772 /sym=Mlc1 /name=Myosin alkali light chain 1/prod=myosin muscle class II essential light chain/func=muscle motor p
193	142538_at	1.5723	FB:FBgn0034341 /sym=CG17531 /name= /prod=glutathione transferase /func=enzyme
194	154346_at	1.5699	FB:FBgn0002973 /sym=numb /name=numb /prod= /func=
195	151800_at	1.5649	FB:FBgn0034229 /sym=CG4847 /name= /prod=cathepsin L-like /func=endopeptidase
196	146489_at	1.5625	FB:FBgn0032777 /sym=CG18576 /name= /prod= /func=
197	153637_at	1.5601	FB:FBgn0033553 /sym=CG12323 /name= /prod= /func=
198	148834_at	1.5601	FB:FBgn0036547 /sym=CG17032 /name= /prod= /func=
199	154884_at	1.5601	FB:FBgn0035763 /sym=CG8602 /name= /prod=permease-like /func=transporter
200	153351_at	1.5576	FB:FBgn0033906 /sym=CG8331 /name= /prod= /func=
201	142343_at	1.5552	FB:FBgn0036911 /sym=CG8660 /name= /prod=FGF-1 intracellular binding protein-like /func=ligand binding or carrier
202	146350_s_at	1.5504	FB:FBgn0032537 /sym=CG18634 /name= /prod= /func=
203	154861_at	1.548	FB:FBgn0029094 /sym=asf1 /name=anti-silencing factor 1 /prod=anti-silencing factor 1 /func=cell cycle regulator
204	152781_at	1.5408	FB:FBgn0004654 /sym=Pgd /name=Phosphogluconate dehydrogenase /prod=/func=phosphogluconate dehydrogenase (decarboxylati
205	146180_at	1.5385	FB:FBgn0032281 /sym=CG17107 /name= /prod= /func=
206	153205_at	1.5385	FB:FBgn0027339 /sym=jim /name= /prod= /func=transcription factor
207	152541_at	1.5385	FB:FBgn0032242 /sym=CG5355 /name= /prod=prolyl oligopeptidase /func=endopeptidase
208	148310_i_at	1.5361	FB:FBgn0035744 /sym=CG8628 /name= /prod=diazepam-binding inhibitor-like /func=enzyme inhibitor
209	154350_at	1.5337	FB:FBgn0033735 /sym=CG8525 /name= /prod=deoxyribose-phosphate aldolase-like /func=enzyme
210	145956_at	1.5337	FB:FBgn0031913 /sym=CG5958 /name= /prod=retinoid binding protein-like /func=ligand binding or carrier
211	143622_at	1.5314	FB:FBgn0010423 /sym=TpnC47D /name=Troponin C at 47D /prod=troponin C /func=calcium binding
212	143310_at	1.5314	FB:FBgn0003134 /sym=Pp1alpha-96A /name=Protein phosphatase 1alpha at 96A /prod=protein serine/threonine phosphatase, PP1, catalytic subunit /func=protein phosphatase type 1 catalyst
213	144913_at	1.5291	FB:FBgn0030366 /sym=CG1490 /name= /prod=ubiquitin thiolesterase /func=endopeptidase
214	153498_at	1.5244	FB:FBgn0027897 /sym=BcDNA:LD03471 /name= /prod= /func=
215	151247_at	1.5244	FB:FBgn0040772 /sym=CG12430 /name= /prod= /func=
216	145970_at	1.5221	FB:FBgn0031931 /sym=CG18589 /name= /prod= /func=
217	142706_at	1.5221	FB:FBgn0036648 /sym=CG4098 /name= /prod= /func=
218	150680_at	1.5198	FB:FBgn0039450 /sym=CG5484 /name= /prod= /func=
219	147903_at	1.5175	FB:FBgn0035089 /sym=CG9358 /name= /prod=
220	143415_at	1.5152	FB:FBgn0004028 /sym=wupA /name=wings up A /prod=troponin I /func=cytoskeletal structural protein
221	154459_at	1.5015	FB:FBgn0030208 /sym=CG2890 /name=Protein phosphatase 4 regulatory subunit 2-related protein /prod=protein phosphatase 4 regul subunit 2-like /func=protein phosphatase
222	143422_at	1.4993	FB:FBgn0004066 /sym=Pros28.1 /name=Proteasome 28kD subunit 1/prod=20S proteasome, alpha4 subunit; multicatalytic endopeptic
223	154281_at	1.4993	FB:FBgn0024846 /sym=p38b /name=antisense Saccharomyces cerevisiae UAS construct /prod=MAP kinase /func=MAP kinase
224	154296_at	1.4948	FB:FBgn0027857 /sym=BcDNA:LD24793 /name= /prod= /func=
225	154675_at	1.4948	FB:FBgn0029978 /sym=CG1515 /name= /prod= /func=

Metal-responsive transcription factor (MTF-1) handles both extremes, copper load and copper starvation, by activating different genes

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From insects to mammals, metallothionein genes are induced in response to heavy metal load by the transcription factor MTF-1, which binds to short DNA sequence motifs, termed metal response elements (MREs). Here we describe a novel and seemingly paradoxical role for MTF-1 in *Drosophila* in that it also mediates transcriptional activation of *Ctr1B*, a copper importer, upon copper depletion. Activation depends on the same type of MRE motifs in the upstream region of the *Ctr1B* gene as are normally required for metal induction. Thus, a single transcription factor, MTF-1, plays a direct role in both copper detoxification and acquisition by inducing the expression of metallothioneins and of a copper importer, respectively.

Supplemental material is available at http://www.genesdev.org.

Received January 31, 2005; revised version accepted March 4, 2005.

Copper is an essential trace element that serves as a catalytic cofactor for several enzymes that are mainly involved in respiration, iron transport, and oxidative stress protection (Puig and Thiele 2002). However, an excess of copper ions can catalyze cytotoxic reactions; thus, every organism must be able to tightly regulate copper levels (Halliwell and Gutteridge 1985). Copper imbalance in humans is the cause of serious diseases, such as Menkes syndrome and Wilson disease, and has also been implicated in Alzheimer's disease and prion-type diseases (Harrison and Dameron 1999; Waggoner et al. 1999; Mercer 2001). Copper homeostasis can be regulated at the level of copper uptake, distribution, chelation, and export (Askwith and Kaplan 1998; Culotta et al. 1999). The cellular proteins that are involved in copper homeostasis, such as importers, exporters, and scavengers, are regulated by different mechanisms including transcriptional activation or repression, changes in protein stability, and the modulation of protein trafficking (Petris et al. 2003; Bertinato and L'Abbe 2004; Lane et al. 2004).

From insects to mammals, heavy metal detoxification is controlled to a large extent by the zinc finger transcription factor MTF-1 (metal response element-binding transcription factor-1, also referred to as metal-responsive transcription factor, or just metal transcription factor) (Westin and Schaffner 1988; Radtke et al. 1993; Langmade et al. 2000; Giedroc et al. 2001; Lichtlen and Schaffner 2001; Zhang et al. 2001). Metal response elements (MREs) of consensus TGCRCNC (where R stands for A or G and N for any of the four bases) are *cis*-regulatory DNA sequences that specifically bind MTF-1 and are essential and sufficient for transcriptional induction upon heavy metal load (Stuart et al. 1985; Westin and Schaffner 1988). Major target genes of MTF-1 are the genes encoding metallothioneins—short, cysteine-rich proteins that have the ability to bind and thereby sequester heavy metals (Kägi and Kojima 1987; Palmiter 1998). In the mouse, *MTF-1* is an essential gene, the knockout of which results in embryonic lethality due to liver degeneration (Günes et al. 1998). The strong up-regulation of the transcription of metallothionein genes upon heavy metal load was abrogated in *MTF-1* knockout cells (Heuchel et al. 1994; Günes et al. 1998). A conditional knockout of *MTF-1* in the mouse liver produced no phenotype in normal laboratory conditions, but mice were more susceptible to cadmium toxicity (Wang et al. 2004). As in the case of mammals, in *Drosophila* a major function of the MTF-1 (dMTF-1) is in the activation of metallothionein genes in response to heavy metal load (Zhang et al. 2001; Egli et al. 2003). There are four metallothionein genes in *Drosophila*, each harboring multiple MREs in their enhancer/promoter region. However, unlike the situation in the mouse, knockout of *dMTF-1* is not lethal in *Drosophila*. The mutant flies ($dMTF\text{-}1^{140\text{-}1R}$) survive well under laboratory conditions but are extremely sensitive to elevated levels of heavy metals including zinc, copper, and cadmium. Consistent with the phenotype, exposure of *dMTF-1* mutants to heavy metal load failed to induce metallothionein genes (Egli et al. 2003; Balamurugan et al. 2004).

In light of the established role of MTF-1 under conditions of heavy metal load, it came as a surprise that in *Drosophila*, *MTF-1* mutants also died at larval stages when challenged with nutritional copper scarcity (Egli et al. 2003). This seeming paradox prompted us to investigate the role of MTF-1 during copper starvation. We conducted microarray analysis and identified the copper importer *Ctr1B* as a potential target gene of dMTF-1. There are three Ctr-type copper transporters in *Drosophila*, namely, Ctr1A, Ctr1B, and Ctr1C (Zhou et al. 2003). Ctr1B function is important during larval stages, where efficient copper uptake is essential for rapid growth. *Ctr1B* knockout flies ($Ctr1B^{3\text{–}4}$) survive well in normal laboratory conditions but are extremely sensitive to nutritional copper scarcity and, to a lesser degree, also to copper load. The sensitivity of the mutants to copper depletion is consistent with the copper uptake function

[*Keywords*: Transcription factor MTF-1; metal response elements; metallothioneins; Ctr1B; copper load; copper depletion]
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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1301805.

of Ctr1B. It was speculated that the sensitivity of the mutants to copper load was due to an inability to mobilize copper to a potential copper-dependent protein or a storage tissue (Zhou et al. 2003).

Here we demonstrate that the lethal phenotype of *dMTF-1* mutants under copper insufficiency conditions is due to the failure of regulating the copper importer Ctr1B. Interestingly, the upstream regulatory region of the *Ctr1B* gene contains MREs that conform to the consensus found in metallothionein genes. By genetic and biochemical analyses we show that these MREs are, however, not used for induction upon copper load, but are essential for the activation of *Ctr1B* by dMTF-1 under conditions of copper scarcity. Thus, we reveal a novel mechanism whereby a single transcription factor, dMTF-1, plays a central role in both copper detoxification and acquisition, by directly activating transcription of metallothioneins and a copper importer, respectively.

Results and Discussion

It was known from the *dMTF-1* knockout study that the *Drosophila* larvae were not only sensitive to excess copper, zinc, and cadmium but also highly sensitive to copper depletion, as tested by supplementing the food with the specific copper chelator bathocuproinedisulfonate (BCS) (Egli et al. 2003). To understand this phenotype, we assessed the transcriptome response in a deletion mutant of the heavy metal regulator dMTF-1 (*dMTF-1*$^{140\text{-}1R}$). A comparison of microarray data from the *dMTF-1* mutant and wild type (WT) larvae revealed that transcripts of one of the copper importers, *Ctr1B*, were reduced in the *dMTF-1* mutant, whereas expression of the related genes *Ctr1A* and *Ctr1C* was not affected (data not shown). A microarray analysis of genes up-regulated in low copper conditions in wild-type *Drosophila*, on the one hand, and genes with decreased expression in the *dMTF-1* deletion mutant in normal food, on the other, revealed *Ctr1B* as the only overlapping gene (Fig. 1A). These findings were confirmed by RNA blotting, which showed in wild-type *Drosophila*, an opposite regulation of the *Ctr1B* gene as compared with a well-characterized target gene of MTF-1, metallothionein A (*MtnA*). While the latter was strongly induced by excess copper in the food, *Ctr1B* was at the same time down-regulated, but induced by copper chelator treatment. In the *dMTF-1* mutant, the *MtnA* transcripts were not detectable at any condition, while *Ctr1B* transcripts were reduced in normal food and could no longer be up-regulated in response to copper chelator treatment (Fig. 1B).

The loss of regulation of *Ctr1B* in the *dMTF-1* mutant prompted us to test whether Ctr1B was responsible for the unexpected sensitivity to copper deprivation of the *dMTF-1* mutant *Drosophila*. For this, we attempted to shortcut the regulation by constitutively overexpressing Ctr1B. Several transgenic fly lines were generated with a *Ctr1B* ORF driven by UAS_{GAL}. The Ctr1B transgenic flies survived well but invariably died if crossed with flies constitutively expressing the Gal4 transcription factor via the actin5c promoter. To test whether this lethality was due to uncontrolled copper import or another effect, we raised the larvae in food with increasing amounts of copper chelator. The results were clear, in that the flies survived only in the presence of high chelator concentrations, while wild-type flies survived under all conditions (Fig. 1C). These observations suggest that the larvae died from copper toxicosis, even in normal food, due to the strong, ectopic expression of Ctr1B. We used the same system to test whether this constitutive expression of Ctr1B could rescue the lethal phenotype of the *dMTF-1* mutant under low copper conditions. *dMTF-1* mutant *Drosophila* are developmentally arrested and die at second or third instar larval stages when the concentration of BCS reaches 50 µM in the food. Strikingly, constitutive Ctr1B expression rescued the developmental arrest and larval lethality of the *dMTF-1* mutants under copper depletion, and several viable *dMTF-1* mutant flies were obtained from food containing 50 or even 100 µM BCS (Fig. 1D; Supplementary Table 1). The rescued *dMTF-1* mutant flies were normal and fertile (Supplementary Fig. 1; data not shown). The constitutive Ctr1B expression and lack of tissue specificity probably prevented a complete rescue of *dMTF-1* mutants in all concentrations of BCS tested.

While these results demonstrated that *Ctr1B* is an essential downstream target gene of dMTF-1 under copper

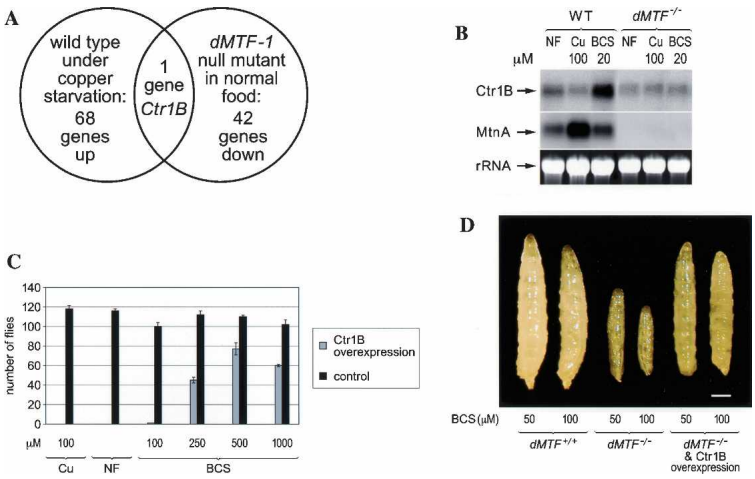

Figure 1. *Drosophila* copper importer *Ctr1B* is a target gene of MTF-1. (*A*) Summary diagram of microarray experiments to identify dMTF-1 target genes in copper depletion (the complete microarray data will be presented elsewhere). Sixty-nine genes were up-regulated more than twofold when wild-type larvae fed with BCS (copper chelator) containing food were compared with larvae fed with normal food (p-value < 0.05). In *Drosophila* larvae lacking *dMTF-1*, 43 genes were down-regulated in normal food, when compared with wild-type controls (p-value < 0.05). *Ctr1B* was the only overlapping gene in these two experimental conditions. (*B*) Regulation of *Ctr1B* upon copper starvation is lost in the absence of *dMTF-1*. RNA blotting analysis of total RNA obtained from third instar larvae at different conditions. (WT) Wild type; (NF) normal food; (rRNA) reference. (*C*) Lethality of Ctr1B overexpression in normal food is rescued by BCS. Ctr1B overexpression, *y w; actin-Gal4/UAS-Ctr1B*; control, *y w; +/UAS-Ctr1B*. *y w; UAS-Ctr1B/UAS-Ctr1B* (homozygous) flies were crossed with *y w; actin-Gal4/+* (heterozygous) flies and were allowed to lay eggs in different food as indicated. The flies from the F1 generation of this cross were counted, and an average from two different experiments is presented. (*D*) Constitutive expression of Ctr1B rescues the developmental arrest and lethality of the *dMTF-1* mutants under copper scarcity. *dMTF-1*$^{+/+}$, *y w* third instar larvae; *dMTF-1*$^{-/-}$, *dMTF-1*$^{140\text{-}1R}$ mutant larvae (lethality under copper starvation occurs at second or third instar stages); *dMTF-1*$^{-/-}$ and Ctr1B overexpression, *y w; dMTF-1*$^{140\text{-}1R}$, *actin-Gal4/dMTF-1*$^{140\text{-}1R}$, *UAS-Ctr1B* larvae. Flies were allowed to deposit eggs in the respective food and larval pictures were taken 5 d after egg deposition. Bar, 1 mm.

starvation, the question remained whether the response was direct or indirect. Inspection of the upstream sequences of the *Ctr1B* gene revealed a cluster of three metal response elements, designated MRE1–MRE3, and a fourth one set apart from them (Fig. 2B). To determine the significance of these MREs, we made a comparison to the several related species of *Drosophila* whose genome sequences are available in the database. We also amplified and sequenced the *Ctr1B* genomic region from *Drosophila virilis* and included these data in our comparison. While the majority of upstream sequences have diverged considerably, the MRE cluster is highly conserved, both regarding the MREs themselves and their flanking sequences, among the four species (Supplementary Fig. 2). The comparisons also revealed that the fourth MRE, which lacks the typical flanking sequences of good MREs, is not conserved in the other *Drosophila* species. To test whether dMTF-1 can bind to the MREs of *Ctr1B*, we conducted electrophoretic mobility shift assay (EMSA) experiments. *Drosophila* S2 cells were transfected with either *Drosophila* MTF-1 or human MTF-1 expression plasmids, and extracts from these cells were tested with radiolabeled oligonucleotides containing MREs from the *Ctr1B* upstream region. Indeed, both MRE1 and an oligo containing the closely spaced MRE2 and MRE3 of *Ctr1B* bound strongly to dMTF-1 and hMTF-1 and are well comparable to the binding of a consensus oligo designated MRE-s (Fig. 2A).

To narrow the region responsible for copper regulation, we tested transgenic flies with deletion constructs driving a fluorescent protein reporter. In one of these, the EGFP coding sequence was fused to the last codon preceding the stop codon of *Ctr1B*, thereby preserving not only the coding sequence but also the introns that might harbor regulatory sequences (AH3). In another construct, the first codon of *Ctr1B* was fused to EGFP (AH2) (Fig. 2B). Transgene expression was found to be strongly induced in the larval gut by BCS-supplemented food (Fig. 3A, panel AH3; for AH2, see Supplementary Fig. 3A). Consistent with the role of Ctr1B in copper import, plasma-membrane-localized green fluorescence was observed in the cells of the larval gut of AH3 transgenic flies (Fig. 3C). Removal of the *Ctr1B* upstream region harboring the MRE1–MRE3 cluster (AH1) had a dramatic effect, in that the reporter gene was no longer inducible by copper depletion (Supplementary Fig. 3A). The quantification of the EGFP transcripts from whole larvae revealed a two- to threefold up-regulation of transcription in BCS-containing food (Fig. 3B; Supplementary Fig. 3B). In line with a role of MTF-1 in *Ctr1B* regulation, there was no green fluorescence from AH2 and AH3 transgenes in the gut of *dMTF-1* knockout larvae (Fig. 3A; Supplementary Fig. 3A). We also generated a genomic deletion of the *Ctr1B* locus by imprecise excision of an adjacent P element. One deletion of 685 bp including the region that harbors the MREs was recovered (Fig. 2B). This *Ctr1B* allele, designated $Ctr1B^{11-19}$, was no longer

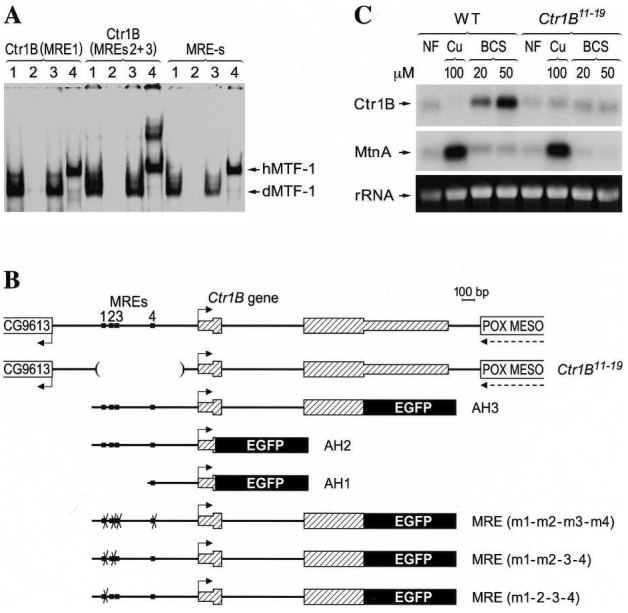

Figure 2. (*A*) MREs of Ctr1B bind MTF-1 very strongly. *Drosophila* S2 cells were transfected with dMTF-1 (lanes *1–3*) or hMTF-1 (lane *4*). (Lanes *2*,*3*) Competitions with 200-fold excess of cold specific and nonspecific competitor oligos, respectively. Human MTF-1, due to its high proline content, migrates more slowly than dMTF-1. Note the double shift with the oligo containing MRE2 and MRE3. (*B*) Schematic view of the *Ctr1B* genomic region, *Ctr1B* mutant $Ctr1B^{11-19}$ and constructs with either wild-type enhancer/promoter region (AH2, AH3), large deletion (AH1), or specific mutations (MRE m1-m2-m3-m4; MRE m1-m2-3-4; MRE m1-2-3-4). (*C*) *Ctr1B* allele ($Ctr1B^{11-19}$) with a deletion in the upstream region shows loss of copper regulation. RNA blotting analysis of total RNA obtained from third instar larvae at different conditions. (WT) wild type, *y w*; (NF) normal food; (rRNA) reference.

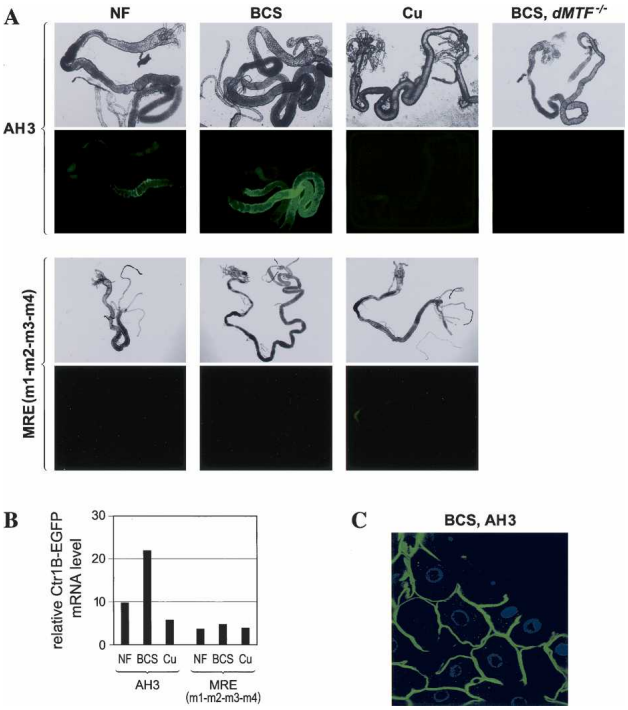

Figure 3. MREs and dMTF-1 are essential for up-regulation of *Ctr1B* upon copper depletion. (*A*) Transgenic flies with AH3 as a reporter show a clear up-regulation of green fluorescence in copper starvation. Induction is lost upon MRE mutations in the reporter constructs (*lower* panel) and also when AH3 transgenics were tested in the *dMTF-1* mutant genetic background (BCS, $dMTF^{-/-}$). For details, see Supplementary Figure 3. (*B*) Quantification of EGFP transcripts by S1 nuclease protection assay from total RNA extracted from *Drosophila* third instar larvae. (*C*) Plasma membrane localization of Ctr1B–EGFP fusion protein in the gut of transgenic larvae when fed with BCS. Nuclei were stained with Hoechst 33342 (blue).

induced by copper starvation (Fig. 2C). For further elucidation of the role of MREs, we constructed several transgenic fly lines that contained point mutations in individual *Ctr1B* promoter MREs (Fig. 2B). The results with transgenic larvae showed that MREs are, indeed, critical for the up-regulation of *Ctr1B* transcription under copper limiting conditions; these specific mutations abolished the expression in low copper, indistinguishable from a deletion of the entire cluster. Even the mutation of a single motif (MRE1) had the same detrimental effect (Fig. 3A; Supplementary Fig. 4).

To assess the biological importance of MREs in *Ctr1B* gene regulation, we tested the ability of the Ctr1B constructs to rescue *Ctr1B*-null mutant flies in low and high copper concentrations. The results confirm the importance of the MREs in the *Ctr1B* gene in that only the Ctr1B–EGFP construct with the wild-type promoter (AH3), but none of the constructs with MRE mutations, rescued the *Ctr1B*-null mutants from lethality in low copper (Table 1). These results lend further credence to a scenario in which *Ctr1B* gene transcription is induced upon copper depletion via upstream MRE sequences and transcription factor MTF-1. As mentioned above, *Ctr1B*-null mutants are also more sensitive to copper load than wild type. The exact reason for this remains to be elucidated; in any case, we find that the high-copper sensitivity can be rescued to a large extent even by a *Ctr1B* transgene lacking the triple MREs. Thus, the main role of these MREs is in copper scarcity, rather than copper load.

Table 1. *MREs are essential for the function of Ctr1B under copper starvation*

		BCS concentration (μM)							
Genotype	NF	10	20	40	80	160	320	640	1280
y w; +/+; +/+	66	67	68	65	66	57	46	36	23
y w; +/+; dMTF$^{140\text{-}1R}$	62	61	55	14	0	0	0	0	0
y w; +/+; Ctr1B$^{3\text{-}4}$	61	56	0	0	0	0	0	0	0
y w; poxMRD; Ctr1B$^{3\text{-}4}$	58	64	60	58	46	43	36	26	12
y w; AH3; Ctr1B$^{3\text{-}4}$	59	60	62	55	41	35	23	2	0
y w; MRE(m1-m2-m3-m4); Ctr1B$^{3\text{-}4}$	61	58	0	0	0	0	0	0	0
y w; MRE(m1-m2-3-4); Ctr1B$^{3\text{-}4}$	67	52	0	0	0	0	0	0	0

		Cu concentration (μM)							
Genotype	NF	10	20	40	80	160	320	640	1280
y w; +/+; +/+	66	65	67	65	66	60	58	52	32
y w; +/+; dMTF$^{140\text{-}1R}$	62	65	60	58	42	16	0	0	0
y w; +/+; Ctr1B$^{3\text{-}4}$	61	59	58	52	34	28	3	2	0
y w; poxMRD; Ctr1B$^{3\text{-}4}$	58	62	61	58	61	52	60	27	19
y w; AH3; Ctr1B$^{3\text{-}4}$	59	64	63	60	52	48	52	24	9
y w; MRE(m1-m2-m3-m4); Ctr1B$^{3\text{-}4}$	61	63	53	55	59	43	32	14	0
y w; MRE(m1-m2-3-4); Ctr1B$^{3\text{-}4}$	67	61	56	61	38	26	12	6	0

Genetic rescue shows the importance of MREs for *Ctr1B* activation under copper starvation. The reporter transgenes that carry the *Ctr1B* gene fused to EGFP with the wild-type promoter and MRE mutations thereof were combined with the *Ctr1B* null mutants. The *poxMRD* transgenic flies seerved as a positive control for the rescue experiment; the poxMRD construct carries the complete *Ctr1B* genomic region including upstream and downstream genes without EGFP fusion. The flies were allowed to lay 200 eggs in the respective food and the parents were removed after 4 d. Results are the mean of three independent experiments and the numbers indicate the percentage of adult flies eclosing.

The results obtained so far demonstrate that dMTF-1 is not only essential for the activation of metallothioneins and other target genes upon heavy metal load, but also to regulate transcription of *Ctr1B* under copper starvation (see also Supplementary Fig. 5). Because in the case of other transcription factors, subtle differences in the DNA-binding site can determine whether the factor interacts with a coactivator or a corepressor (e.g., Dostert and Heinzel 2004), we considered it possible that the MRE motifs of *Ctr1B* themselves could bring about transcriptional induction at low copper. To this end, we generated two types of reporter transgenes with a synthetic "minipromoter," one containing the four MRE motifs from the *Ctr1B* gene with hardly any intervening sequences, arranged in tandem arrays, and another one where only MRE1 was multimerized to four copies (Fig. 4A). We compared these two reporter transgenes with a similar synthetic minipromoter, which contains a tandem array of MRE motifs derived from the metallothionein B (*MtnB*) gene (Zhang et al. 2001). Interestingly, all three reporter transgenes behaved like a genuine metallothionein promoter: They were strongly induced when the larvae were fed with copper, but were not responsive to low copper (Fig. 4B; data not shown). Also in cell culture, all three reporter constructs were robustly induced by copper treatment (Fig. 4C). Thus, the *Ctr1B* MREs on their own are not sufficient to confer transcriptional induction upon copper depletion, but rather respond to metal load. This suggests that sequences in addition to MREs in the *Ctr1B* enhancer/promoter region contribute to the regulatory characteristics of that gene.

How could one transcription factor exert two diametrically different functions? One possibility could be that this special architecture of the MREs in the *Ctr1B* enhancer/promoter facilitates cooperative binding between dMTF-1 and a hypothetical copper-dependent repressor protein. Under normal conditions, this repressor would be partially removed, resulting in a moderate expression, while under copper starvation it would dissociate from dMTF-1 completely, yielding higher expression. Further experiments will be required to elucidate the exact mechanism of *Ctr1B* activation via dMTF-1 under conditions of copper starvation.

How do other organisms handle copper excess and copper starvation? In the yeast *Saccharomyces cerevisiae*, the two extremes require different transcription factors. The homologs of Ctr1 that import copper are activated upon copper starvation by the Mac1 transcription factor (Yamaguchi-Iwai et al. 1997); the activation of metallothionein genes upon copper load is driven by the transcription factor Ace1 (Thiele 1988; Winge 1998; Rutherford and Bird 2004). In mammals, there are two Ctr homologs, Ctr1 and Ctr2. Neither of them is apparently regulated at the level of transcription by copper availability (Lee et al. 2001, 2002), and we also did not find any MREs in their enhancer/promoter region (data not shown). In conclusion, the major role of MTF-1 is to handle heavy metal load; accordingly, MREs are found in the metallothionein genes and other metal-responsive genes from insects to mammals. In contrast, regulation of the *Ctr1B* copper importer via MREs/MTF-1 appears to have evolved specifically in *Drosophilidae* as an efficient way to cope with copper starvation. This represents a novel regulatory mechanism in which one and the same transcription factor serves as an activator of different genes in response to opposite environmental conditions.

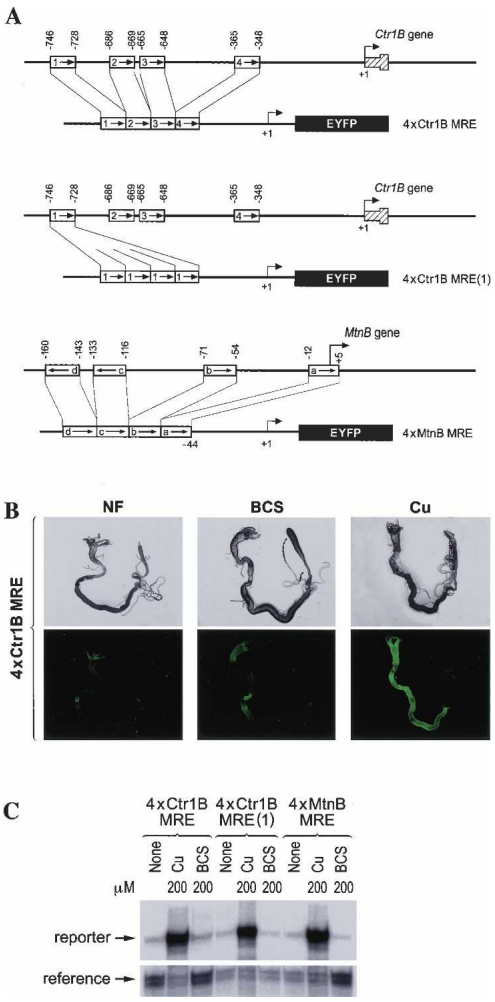

Figure 4. MRE motifs from metallothionein *MtnB* and also from copper importer *Ctr1B* confer high copper induction. (*A*) Minipromoters containing four tandem MREs of the *Ctr1B* upstream region (*upper* construct), four copies of *Ctr1B* MRE1 (*middle* construct), or four MREs from the *MtnB* upstream region (*lower* construct) fused to the minimal heterologous promoter from the *hsp70* gene. (*B*) Minipromoter containing four MRE motifs from the *Ctr1B* gene up-regulates the reporter transgene in high copper, similar to the metallothionein promoter. (*C*) Minipromoters containing four MREs of the *Ctr1B* upstream region, four copies of *Ctr1B* MRE1, or four MREs from the *MtnB* upstream region were assembled into an OVEC reporter construct. The constructs were transfected into *Drosophila* S2 cells, and total RNA was subjected to S1 nuclease protection assay. (Reference) Endogenous *tubulin* α*1* transcripts.

Materials and methods

Fly food, fly stocks, and genetics

Flies were raised on standard cornmeal-based food; 200 µM BCS or 200 µM copper was supplemented to the food for all the fluorescence analyses except for the experiment with the AH3 and AH2 transgene in a *dMTF-1* mutant background, where 40 µM BCS was used. Both AH3 and AH2 are well inducible at 40 µM BCS (data not shown). The homozygous null allele for *dMTF-1* (*dMTF-1*$^{140\text{-}1R}$) is indicated throughout the manuscript as *dMTF-1*$^{-/-}$. The EP(3)0833 line, which harbors a single P element 800 bp upstream of the *Ctr1B* transcription start site, was obtained from the Bloomington Stock Center. To generate a *Drosophila Ctr1B* promoter deletion (allele *Ctr1B*$^{11-19}$), an imprecise P-element excision strategy was used as described in Zhou et al. (2003). A complete list of fly stocks generated is included in the Supplemental Material.

DNA constructs

See Supplemental Material.

GFP expression analysis and microscopy

For the EGFP or EYFP reporter analysis, flies were allowed to deposit eggs in the food and raised until third instar larvae. The larval gut was dissected and analyzed under a Leica DRB fluorescence stereomicroscope. The images in Figure 3A are magnified 5×, and all the other larval gut images were made at 2.5× magnification. For the subcellular localization of Ctr1B, *Drosophila* larval gut was analyzed at 63× magnification using a LEICA TCS SP spectral confocal microscope.

Cell culture and transient transfection assay

Drosophila S2 cells were grown at 25°C under standard culture conditions. Various OVEC reporter constructs driven by *Drosophila* promoters were transfected together with an expression vector for dMTF-1 driven by the *Drosophila* actin5c promoter using the calcium-phosphate coprecipitation method (Westin et al. 1987). Seventy-two hours post-transfection, cells were treated with the indicated concentrations of $CuSO_4$ or BCS and incubated for another 24 h before harvesting.

RNA extraction, S1 nuclease protection assay, and RNA blotting

Flies with various genotypes were grown in normal, BCS-supplemented, or copper-supplemented food, and the third instar larvae were harvested for total RNA extraction using the TRIzol reagent (Invitrogen). The S1 nuclease protection assay was performed using 100 µg of total RNA as described previously (Weaver and Weissmann 1979). The gels were developed using a PhosphorImager, and bands were quantified using ImageQuaNT software. For quantification of EGFP transcripts, endogenous actin5c was used for normalization. RNA blotting experiments were performed using the *Ctr1B* and *MtnA* cDNA as ^{32}P-labeled probes. rRNA (reference) was stained with ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Transient transfections in *Drosophila* S2 cells were carried out as mentioned above. The nuclear extracts were prepared and EMSA was performed as described previously (Radtke et al. 1993). Binding reactions were performed by incubating 25 fmol of ^{32}P end-labeled, a 28-bp-long oligonucleotide containing the MRE1 motif from *Ctr1B* promoter, a 40-bp-long oligonucleotide containing the closely spaced MRE2 and MRE3 from *Ctr1B*, or a 31-bp-long DNA oligonucleotide containing an MRE core consensus sequence TGCACAC designated MRE-s (Radtke et al. 1993) as a positive control for MTF-1 binding. For competition experiments, 5 pmol of unlabeled oligo (either specific or nonspecific) was added to the reaction mixture prior to addition of the extracts. For complete oligonucleotide sequences, see Supplemental Material.

Database searches and computer analysis of the sequences

Database homology searches were carried out using the University of California, Santa Cruz, blat server (http://www.genome.ucsc.edu). The alignment was done in CLUSTALW.

Acknowledgments

We are greatly indebted to Fritz Ochsenbein for the preparation of figures. We thank Antonia Manova and Bruno Schmid for technical assistance. We also thank Jianming Chen and Markus Noll for the poxMRD flies, Hajime Takeuchi and Janis Bennion for critical reading of the manuscript, and Ken Cadigan for helpful advice. This work was supported by the Swiss National Science Foundation, the Kanton Zürich, and a grant from the United States National Institutes of Health (GM62555) to D.J.T.

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Supplementary figure legends

Figure 1. Constitutive expression of Ctr1B rescues the developmental arrest and lethality of the *dMTF-1* mutants in copper scarcity. *dMTF-1*$^{+/+}$, *y w*; *dMTF*$^{-/-}$ & Ctr1B overexpression, *y w; dMTF-1*$^{140\text{-}1R}$*, actin-Gal4/dMTF-1*$^{140\text{-}1R}$*, UAS-Ctr1B*. The flies were raised in 100 μM BCS and all of them were one day old when the picture was made.

Figure 2. Complete alignment of *Ctr1B* enhancer/promoter sequences from four *Drosophila* species. 1500 bases upstream of the translational start site were used for alignment. The transcription and translation start of *Ctr1B* and the upstream gene *CG9613* are indicated. The *D. melanogaster* and *D. yacuba* are closely related species. *D. pseudoobscura* is separated by approximately 25 million years from *D. melanogaster*. *D. virilis* is separated by no less than 40 million years from *D. melanogaster* (Lemeunier et al. 1986; Grimaldi 1990; Russo et al. 1995).

Figure 3. Deletion of the upstream region that contains the MREs abolishes the transcriptional induction of *Ctr1B* under copper starvation. (A) Transgenic flies with AH1 or AH2 as reporters were allowed to deposit eggs in the respective food. Third instar larval guts were dissected and inspected for green fluorescence. (B) Quantification of EGFP transcripts from the total RNA extracted from *Drosophila* third instar larvae.

Figure 4. MREs are essential for the transcriptional induction of *Ctr1B* in copper starvation. Larvae carrying reporter transgenes with mutations in the MRE sequences were allowed to develop in the respective food and analyzed.

Figure 5. A schematic representation shows that the transcription factor dMTF-1 directly activates the *Ctr1B* gene in copper starvation and metallothionein genes in copper load through MREs.

Supplementary Table 1. Flies were allowed to deposit 150-200 embryos in the respective food and the parents were removed after 4 days. (a, b) Embryos are derived from stocks of the indicated genotype. (c) *UAS-Ctr1B* transgenic flies in the *dMTF-1* mutant background were crossed with *actin-Gal4* flies also in the *dMTF-1* mutant background and allowed to deposit embryos in the respective food. The frequency of survival to adulthood for the indicated genotype was calculated by dividing the number of F1 progeny with the expected number according to Mendel`s law. The results presented are the mean percentage of progeny from three independent experiments.

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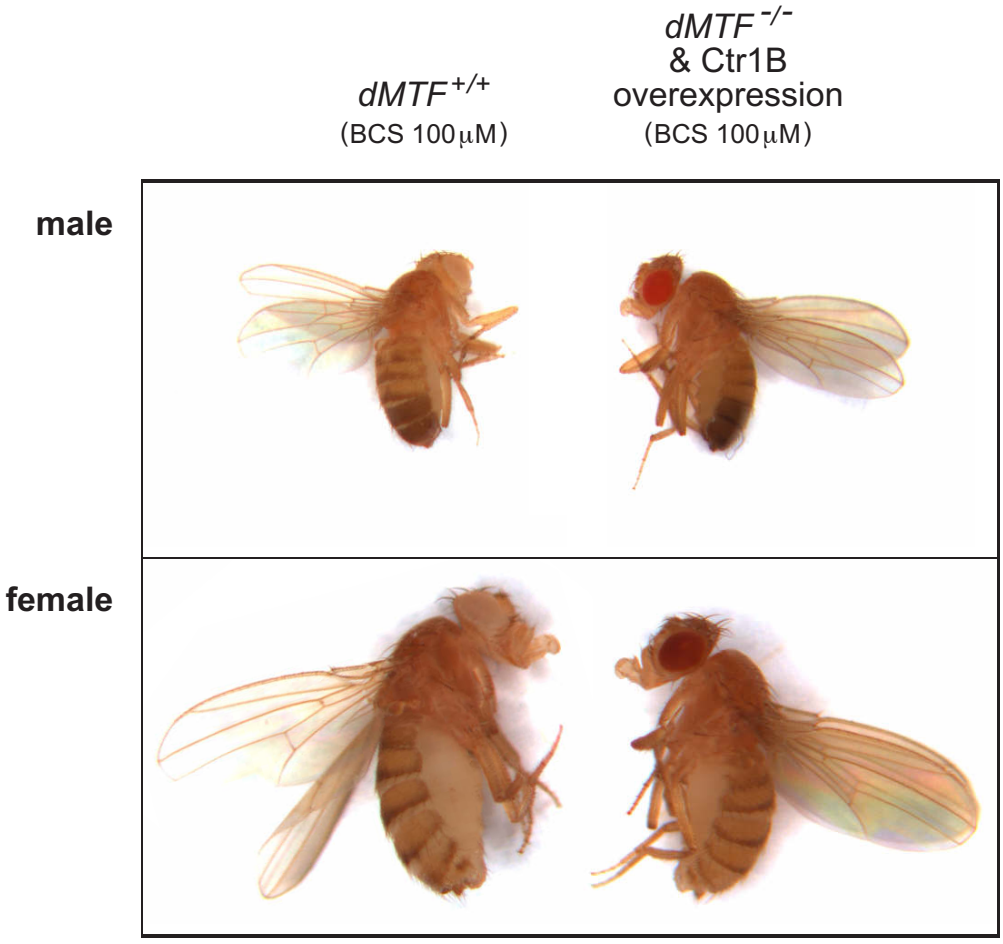
Supplementary Figure 1



Supplementary Figure 2: Comparison of Ctr1B upstream region
(-1500 bases upstream of Ctr1B translational start)

```
D. melanogaster    1 CCTGCAGCCGGGACTTATCCCAAGTGCTCCAATCTCCAATCAG--AACA-
D. yakuba          1 ------------------------------------ATCAG--AACA-
D. pseudoobscura   1 ------------------------TTATTGGATTTTGCTTAAA--AAAA-
D. virilis         1 ---TTTTTTAGCAACTATTATATGTATTTGAAAAAAAATTAAGCAAACAT
consensus          1                                       T A   AA A

D. melanogaster   48 CGC----GCTGGTTGCCG-----GGGCA-GCATGTGTTTTGTTGTTGC-C
D. yakuba         10 CGC----GCTGGTTGCCG-----GGGCA-GCATGTGTTTTGTTGTTGC-C
D. pseudoobscura  24 GG--------GTTTAGCGAAAAAGGTCAAGCAAATGAAACGTAATAAAAC
D. virilis        48 CGCTATCGCTGTTTAAAT---------ATTAAAGTAAAATATCGTTTACC
consensus         51  G        G TT             A   A  T      T  T    C

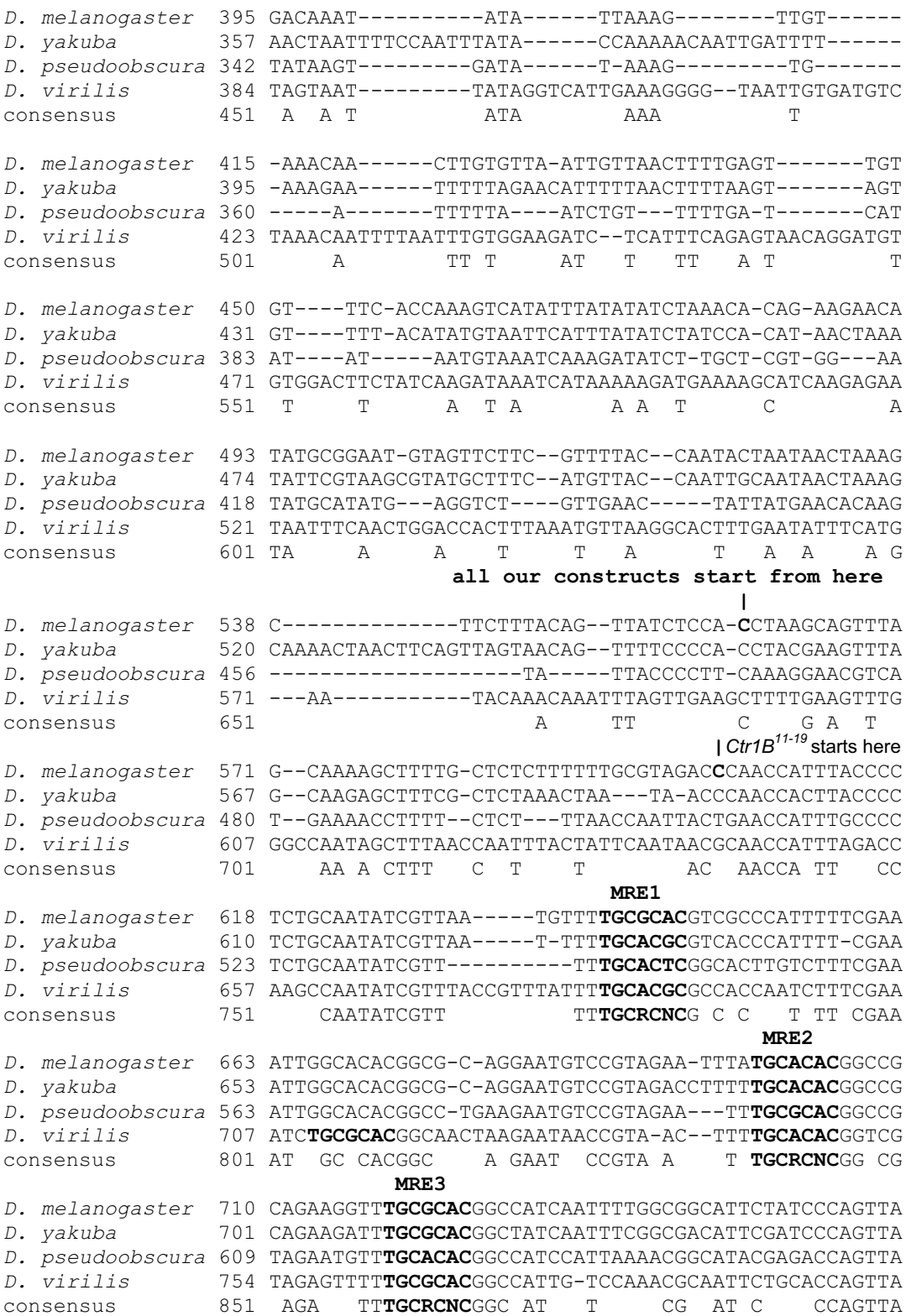
D. melanogaster   87 GCC-GCTGCATAAGAGCTGC--GGAAGTGCC---TAGCGCTCTGGAGTCG
D. yakuba         49 GCC-GCTGCATAAGAGCTGC--GGAGGTGTC---GTGCGCTCTGGAGTCG
D. pseudoobscura  66 GTA-AGAGAAAAATTGTTGC----AATTGTTCAATTTCTTTATTCCGTTG
D. virilis        89 TAATGCTGCATACGATATTCTAGAAAATATC------CCTACTT---TTG
consensus        101        G A A     T C    A  T         C    T    T G
                                      ⟵ATG of CG9613
D. melanogaster  131 CAGGTGTCGAAGCGCA-T----ACATTGC-GATTTGCCTTGCA----CTA
D. yakuba         93 CAGGTGTCGTAGCGCA-T----ACATTGC-GATTTGCCTTGCA----CTA
D. pseudoobscura 111 AATAATTC-TACCTAACT----AAAA--C-CATTAGCTCTGCC----
D. virilis       130 CATA-GGC--AACTGAGTTGCCACATTGCTGCTTTGATTTTTAAATGCTG
consensus        151  A     C  A C  A T    A A   C   TT G   T       C

D. melanogaster  171 GCACCTTCAACGATT-AAAG--CATTAAGTCACACGATGTTAAGTTGTAT
D. yakuba        133 GCACCTGGAATAATT-TAAG--CATTAAGTCACTCTATATTAAGTGGTAT
D. pseudoobscura 147 --AC-----ATAATT-CTAGGCCGTTAA-TGAATCAATTTTC--TATTAT
D. virilis       177 CCAC-----ATTATTGAAAG------AA---AAACGTTTTACATTTGTAT
consensus        201   AC     A  ATT   AG      AA   A  C  T T    T  TAT

D. melanogaster  218 TTGTTCGT---AAA-CTCACAGT-----CAAAAAATG-GC----AAGATG
D. yakuba        180 TTATTCGT---TAA-CTCACAAT-----AAAAAAATG-GC----AAGATG
D. pseudoobscura 186 T-GTAAGTGAATAA-AACAAGGTTTAAGCAAAAAAGG-TC----AACAAA
D. virilis       213 G-GTAAGGC--TAAGAACAAGATTT---CAAATCACGCGCTCTTAACAGA
consensus        251    T  G     AA   CA   T      AAA  A G  C    AA A
                      ↰┐Transcription start of CG9613
D. melanogaster  254 TTTA--ATGG-AACTTTGGTTGGGAATGTCAAAGGGCTGCCATCCGGGTA
D. yakuba        216 TTTT--GTGG-AACTTTGGTTGGGAATGTCGAAGCGATGCCATCCGGGTG
D. pseudoobscura 229 AAAACAGTGGCAACGTAAGT--G-AGTATGAAA----TGTCA--CG---T
D. virilis       257 TTCG--ATGG--ACGTTCTCA---AATTCGAAAG------CATAATTCTA
consensus        301        TGG  AC T        A T    AA       CA

D. melanogaster  301 AAGGCAGACAAAGAGTTGCCGGATAGATGAAA---AAACAATA---TGAT
D. yakuba        263 GAGGTAGGCGAAGAGATGCCAGACAGATGAAA---AATGATTT---TTAT
D. pseudoobscura 267 CACGTAACGTATGAAATGGCAGTTAAAT---------TCAATA---TTGT
D. virilis       294 GAAATAATTTTAGCTTTT---ATTAAATTATATTTATTGACTTCTTTTAT
consensus        351  A   A      G   T       A AT           A T    T  T

D. melanogaster  345 CCGGTGGCACTGCTGCATTTGGCGATAAAAATTTGAATTTAAGTTTCAAA
D. yakuba        307 CTGGTGGCACCGCCAGACTTGCCGCTAAAATTACGAATTTAAATCCCAAA
D. pseudoobscura 305 TTCGCAGAA--GGTTCTCTT---------ATTTTGAATT--ATTTCCAGA
D. virilis       341 ATTTCAAAAGTG------TTACCGA-AAAAATAGTAATTATAGTAACAAA
consensus        401         A  G      TT         A T   AATT  A T  CA A
```


D. melanogaster 395 GACAAAT----------ATA------TTAAAG--------TTGT------
D. yakuba 357 AACTAATTTTCCAATTTATA------CCAAAAACAATTGATTTT------
D. pseudoobscura 342 TATAAGT---------GATA------T-AAAG---------TG-------
D. virilis 384 TAGTAAT---------TATAGGTCATTGAAAGGGG--TAATTGTGATGTC
consensus 451 A A T ATA AAA T
D. melanogaster 415 -AAACAA------CTTGTGTTA-ATTGTTAACTTTTGAGT-------TGT
D. yakuba 395 -AAAGAA------TTTTTAGAACATTTTTAACTTTTAAGT-------AGT
D. pseudoobscura 360 -----A-------TTTTTA----ATCTGT---TTTTGA-T-------CAT
D. virilis 423 TAAACAATTTTAATTTGTGGAAGATC--TCATTTCAGAGTAACAGGATGT
consensus 501 A TT T AT T TT A T T
D. melanogaster 450 GT----TTC-ACCAAAGTCATATTTATATATCTAAACA-CAG-AAGAACA
D. yakuba 431 GT----TTT-ACATATGTAATTCATTTATATCTATCCA-CAT-AACTAAA
D. pseudoobscura 383 AT----AT-----AATGTAAATCAAAGATATCT-TGCT-CGT-GG---AA
D. virilis 471 GTGGACTTCTATCAAGATAAATCATAAAAAGATGAAAAGCATCAAGAGAA
consensus 551 T T A T A A A T C A
D. melanogaster 493 TATGCGGAAT-GTAGTTCTTC--GTTTTAC--CAATACTAATAACTAAAG
D. yakuba 474 TATTCGTAAGCGTATGCTTTC--ATGTTAC--CAATTGCAATAACTAAAG
D. pseudoobscura 418 TATGCATATG---AGGTCT----GTTGAAC-----TATTATGAACACAAG
D. virilis 521 TAATTTCAACTGGACCACTTTAAATGTTAAGGCACTTTGAATATTTCATG
consensus 601 TA A A T T A T A A A G
all our constructs start from here
|
D. melanogaster 538 C--------------TTCTTTACAG--TTATCTCCA-CCTAAGCAGTTTA
D. yakuba 520 CAAAACTAACTTCAGTTAGTAACAG--TTTTCCCCA-CCTACGAAGTTTA
D. pseudoobscura 456 --------------------TA-----TTACCCCTT-CAAAGGAACGTCA
D. virilis 571 ---AA-----------TACAAACAAATTTAGTTGAAGCTTTTGAAGTTTG
consensus 651 A TT C G A T
| Ctr1B11-19 starts here
D. melanogaster 571 G--CAAAAGCTTTTG-CTCTCTTTTTTGCGTAGACCCAACCATTTACCCC
D. yakuba 567 G--CAAGAGCTTTCG-CTCTAAACTAA---TA-ACCCAACCACTTACCCC
D. pseudoobscura 480 T--GAAAACCTTTT--CTCT---TTAACCAATTACTGAACCATTTGCCCC
D. virilis 607 GGCCAATAGCTTTAACCAATTTACTATTCAATAACGCAACCATTTAGACC
consensus 701 AA A CTTT C T T AC AACCA TT CC
MRE1
D. melanogaster 618 TCTGCAATATCGTTAA-----TGTTTTGCGCACGTCGCCCATTTTTCGAA
D. yakuba 610 TCTGCAATATCGTTAA-----T-TTTTGCACGCGTCACCCATTTT-CGAA
D. pseudoobscura 523 TCTGCAATATCGTT----------TTGCACTCGGCACTTGTCTTTCGAA
D. virilis 657 AAGCCAATATCGTTTACCGTTTATTTTGCACGCGCCACCAATCTTTCGAA
consensus 751 CAATATCGTT TTGCRCNCG C C T TT CGAA
MRE2
D. melanogaster 663 ATTGGCACACGGCG-C-AGGAATGTCCGTAGAA-TTTATGCACACGGCCG
D. yakuba 653 ATTGGCACACGGCG-C-AGGAATGTCCGTAGACCTTTTTGCACACGGCCG
D. pseudoobscura 563 ATTGGCACACGGCC-TGAAGAATGTCCGTAGAA---TTTGCGCACGGCCG
D. virilis 707 ATCTGCGCACGGCAACTAAGAATAACCGTA-AC--TTTTGCACACGGTCG
consensus 801 AT GC CACGGC A GAAT CCGTA A T GCRCNCGG CG
MRE3
D. melanogaster 710 CAGAAGGTTTGCGCACGGCCATCAATTTTGGCGGCATTCTATCCCAGTTA
D. yakuba 701 CAGAAGATTTGCGCACGGCTATCAATTTCGGCGACATTCGATCCCAGTTA
D. pseudoobscura 609 TAGAATGTTTGCACACGGCCATCCATTAAAACGGCATACGAGACCAGTTA
D. virilis 754 TAGAGTTTTTGCGCACGGCCATTG-TCCAAACGCAATTCTGCACCAGTTA
consensus 851 AGA TTGCRCNCGGC AT T CG AT C CCAGTTA

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D. melanogaster  760 GAAATAGCACAGTAAGAAATAACACTCTAGGAAAGCCAAGTGG-ACTAAG
D. yakuba        751 GAAATAGCACAGTAAGAAATAACACTCTACGAAAGCCAAGTGG-ACTAAG
D. pseudoobscura 659 GAAATA-----------A----AACTCTTTGAAAGCCAAGTGGGACTAAG
D. virilis       803 GAAACA----------AA-----ACATTAGCAAAGCCAAGAGGGCCTAAT
consensus        901 GAAA A           A     AC  T   AAAGCCAAG GG  CTAA

D. melanogaster  809 GCAGACCTCCGGCGATC-CGGCGGCCGACGGTTGCTATCGCTCCCATGCC
D. yakuba        800 GCAGACCTCCGGCGATC-CGCCGACCGACGGTTGCTATCGCTCCCGTGCC
D. pseudoobscura 694 GCAGACAACCGGCAAAATCGCCGCCCGACGGTTGCTATCGCTCC--AGCC
D. virilis       838 GCAGACATCAAGCAAATTCGCTCGTCGATGGTTGCTATCGCTCG------
consensus        951 GCAGAC  C  GC A   CG     CGA GGTTGCTATCGCTC

D. melanogaster  858 GGAATATGGGCATATACCTACGTATGAATGTACTCGACTAGAGC--CATA
D. yakuba        849 GGTATATGGGCATATACCTACTTATGAACGTACCCGGCTAGAGC--CATA
D. pseudoobscura 742 AGT---TGCCCATGTATGTACCCATGTAGGTGCGCGTATATATCAGCGGA
D. virilis       882 AGAA-ATATGCACGTACATACATGGGAACATACT------TAGCTGCGTA
consensus       1001  G    T   CA  TA  TAC    G A  T C        A C  C  A

D. melanogaster  906 AAACCGTGA---TCGAAGCGAAG--AAATAATGGAA-TTATTTCAACATC
D. yakuba        897 AAACCATGA---TCGAACGGAAG--AAATAATGGAA-TAATTTCAACATC
D. pseudoobscura 789 ATACCATAAAGTTCAACTCGAAGGAAAATAGAGCAAATCGTTTAAACACA
D. virilis       925 CAA---TAA----------------A-ATA----AA-TACTTTGACCA--
consensus       1051   A   T A                A ATA    AA T  TTT A CA

D. melanogaster  950 ATGTATTACAGCAACAAT-TTCAGGGGG----TTGTA----TTAGCCCAT
D. yakuba        941 ACGTATTACAGCAACAAT-TTCAGGGGG----TTGTA----TTAGCCCAT
D. pseudoobscura 839 TTGAGATGCAGCAACAAT-TTCGTTAGC----TCGTG----TTGGCCAAT
D. virilis       948 ----ATTAC--CAATAATCTTAATTACCACACTTGTGAACTTCAGAGAAT
consensus       1101       T C  CAA AAT TT           T GT     T  G   AT
                                                     MRE4
D. melanogaster  991 G-ATTGATAATCTGAATTGCAATAAAT---TCTGCAC--ACGAATCGATT
D. yakuba        982 G-ATTGATAATCTGAGTTGCAATAAAT---TCTGCAC--ACGAATCGATT
D. pseudoobscura 880 G-AT----AATCTTAATTGCAATAAATAAGTCTGCACTTAAGAAT--ATT
D. virilis       992 CCATA---AATCAATATTGAAATAGAA---TTTGCTCA-AGGAAA--AAA
consensus       1151   AT    AATC    TTG AATA A    T TGC C  A GAA   A

D. melanogaster 1035 GTCGAACCTTTGCCATGAGTCC-ATAAATTAAGCTGCAAACCGCAGAGAA
D. yakuba       1026 GTCGAACCTTTGCCATCAGTCC-ATAAATTAAATGGCAAATCGAAGGGAA
D. pseudoobscura 923 CCACAACCTTTGGTTT--GCCCTATAAATCACTCTGT-----GTAGAATG
D. virilis      1033 ATACAAGTTT--C-AT--GCTCTACCAAGTA---TCC------------A
consensus       1201     AA  TT     T  G  C A  AA  A

D. melanogaster 1084 ACCCACGACGCATCAATTATGGATTTTA------TCT---------CTCG
D. yakuba       1075 GCCCAA---GGATCAATGATGGGTTTTA------TCT---------CTCG
D. pseudoobscura 966 GGCTATT--GGTTTTTTTTTGGTGTAAAGTACAGTCTGATGATGACCTTG
D. virilis      1063 GGCCA----G-ATCACAGCCAG-------------CTGCA------CACA
consensus       1251   C A    G  T        G             CT         C

D. melanogaster 1119 AGATAAAGATCTGCAACT--------GATACCCAGAA--TATA-------
D. yakuba       1107 AGATAAAGATCTGCATATCCAT--AAGATATCCAGAA--AATA-------
D. pseudoobscura1014 ATCTGAA-ATATGCAAATCTACCAAAGGTAAAAAGATG-AATAGCTGTTT
D. virilis      1089 GCATGCAGATCTGCAACT--------GATAAATAGATGGACTA-------
consensus       1301    T  A AT TGCA  T        G TA   AGA     TA

D. melanogaster 1152 ----TCTT-----TTGATCTGACCTCT---------AACACTG------A
D. yakuba       1146 ----TCTT-----TTGATTTGCCCTGA---------AACAATG------G
D. pseudoobscura1062 TTATTCTTC----TTTATCTTATCTTATCTTGTGGGAAAGATGTATTACA
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D. virilis       1124 ----TCATTAACATTTATTTGGGTTTC-C-------ATCTCAGT-----T
consensus        1351     TC T     TT AT T    T           A     G

D. melanogaster 1178 GATC---------T---G------CATGCATTTATGTTTATT----TACA
D. yakuba        1172 GATC---------TCT-G------AATGCATTTATGTTTATT----AACA
D. pseudoobscura1108 GATTATAATCATGTTT-GGTGATAAATGTATTTATATCTATTCGTGTTTA
D. virilis       1157 GCTC---------TCTCGCT----CACTC-TCTCTCTCTCTCC---CACA
consensus        1401 G T          T   G       A    T T T T T T        A

D. melanogaster 1206 TGACTGTATCT----GGGCTGCCT------TT---CT-TAGTG--TGGGG
D. yakuba        1202 AAGCTGTATCT----GGGTTGGCA------TT---CT-TATTC--AGGGG
D. pseudoobscura1157 TATATGTATAT----CGG--GCCA------TTACTCT-TGCTC--AGTGT
D. virilis       1190 CACCTGTTACTCAATGGG--GTCAACATCGTTGGGCTCTCCTCGCA TGCG
consensus        1451     TGT   T     GG  G C       TT   CT T  T

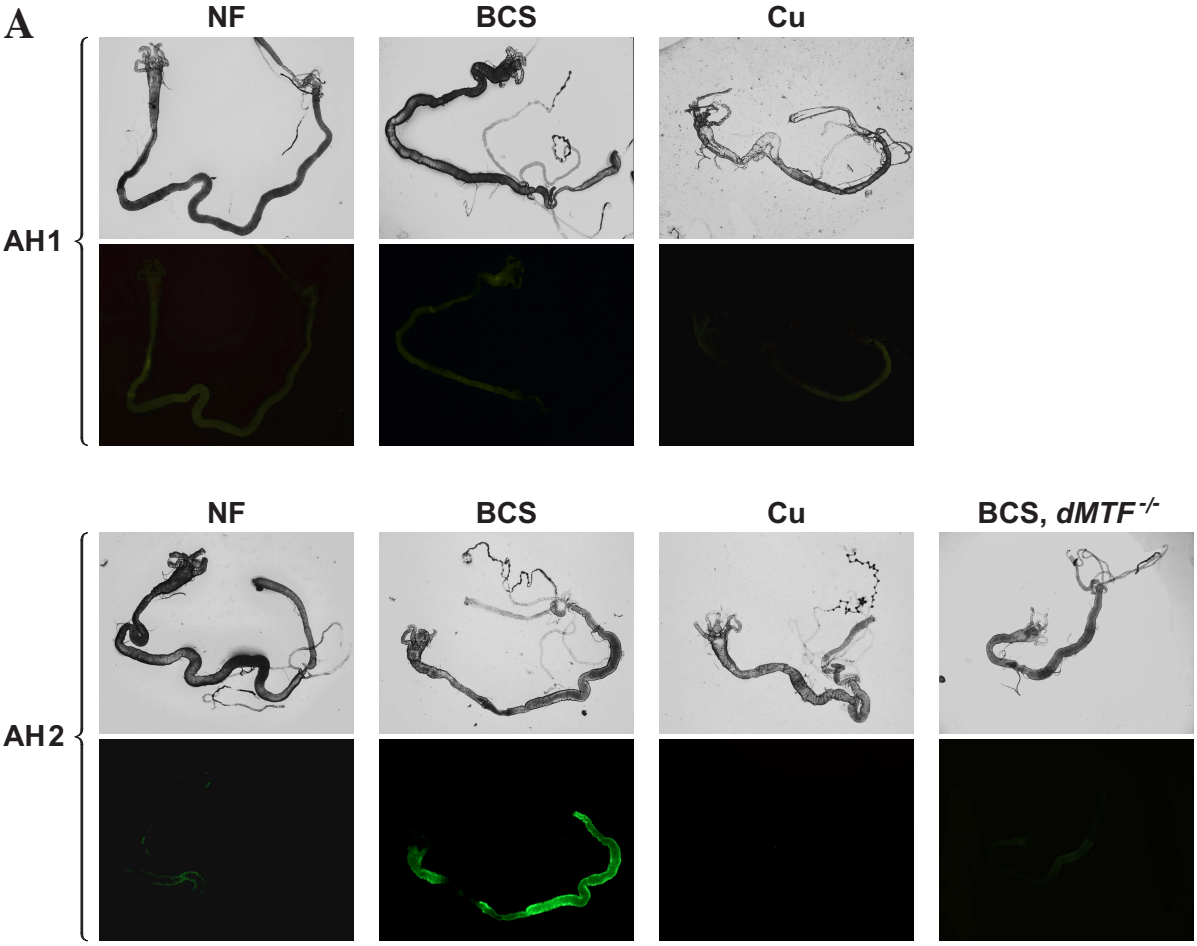
D. melanogaster 1240 ------CGACTATC-----------GTTGGTATCG---------------
D. yakuba        1236 ------CCACTATC-----------GTAGGTATCG---------------
D. pseudoobscura1192 AAAGACCCCCTCTCTGGGCTGGGCTGGTGATATCGGCGCCTGCAACCTGC
D. virilis       1238 -------CGCTATC-----------GATGATATCGCC------AGCGTGC
consensus        1501          CT TC           G  G TATCG
                                        Ctr1B11-19 ends here |
D. melanogaster 1258 ---CCGCTACTGGGTGATAACGCTC-TTTCCCATTGCCCATAGATAAGCG
D. yakuba        1254 ---CGGCTACTGGGTGATAACGCTC-TTTCCCAGTGCCCATAGATAAGCG
D. pseudoobscura1242 TGCCCGCTACCAGTTGATAACGTGAATTTCCCATTTCTCAGAGATAAGCC
D. virilis       1264 ACTCCGCG-CTTGCTGATAACACAA-------ATTTCTCAGAGATAAGCG
consensus        1551    C GC  C  G TGATAAC           A T C CA AGATAAGC

D. melanogaster 1304 CACTTTCCTATCGCCGCGTCGGGGG-ATTAGGTGC-GATTTT-CTTTAAA
D. yakuba        1300 CACTTGCCTATCGCCGCGTCGGGGGGATTAGGGGC-GATTTT-CTTTAAA
D. pseudoobscura1292 CACTTGCCTATCGTCTCGACGGGG--ATTAGGGTC-GATTTTTCTTTAAA
D. virilis       1306 CACCGGC-TATCGCCTTGGCGGG---ATTAGTGTCAGGTTTT-CTTTAAA
consensus        1601 CAC   C TATCG C  G CGGG   ATTAG   C G TTTT CTTTAAA
                                        Transcription start of Ctr1B
                                                ┌►
D. melanogaster 1351 AAACCACTCGCGAGCAGCGGGTTTCAAGCAGTCAACGCTCTGCTCTCAGC
D. yakuba        1348 AAACCACTCGCGAGCAGCGGCTTTCAAGCAGTCAACGCTCTGCTCTCAGC
D. pseudoobscura1339 AAACAACTCACGACCATATGGGGGCAA-CAGTCGACGCTTCGCTCTCAGT
D. virilis       1351 AAACAGCATACGTTCGTCTG-TCCCAAGCAGTCAACGTTACGCTCTCAGC
consensus        1651 AAAC  C   CG  C    G    CAA CAGTC ACG T  GCTCTCAG

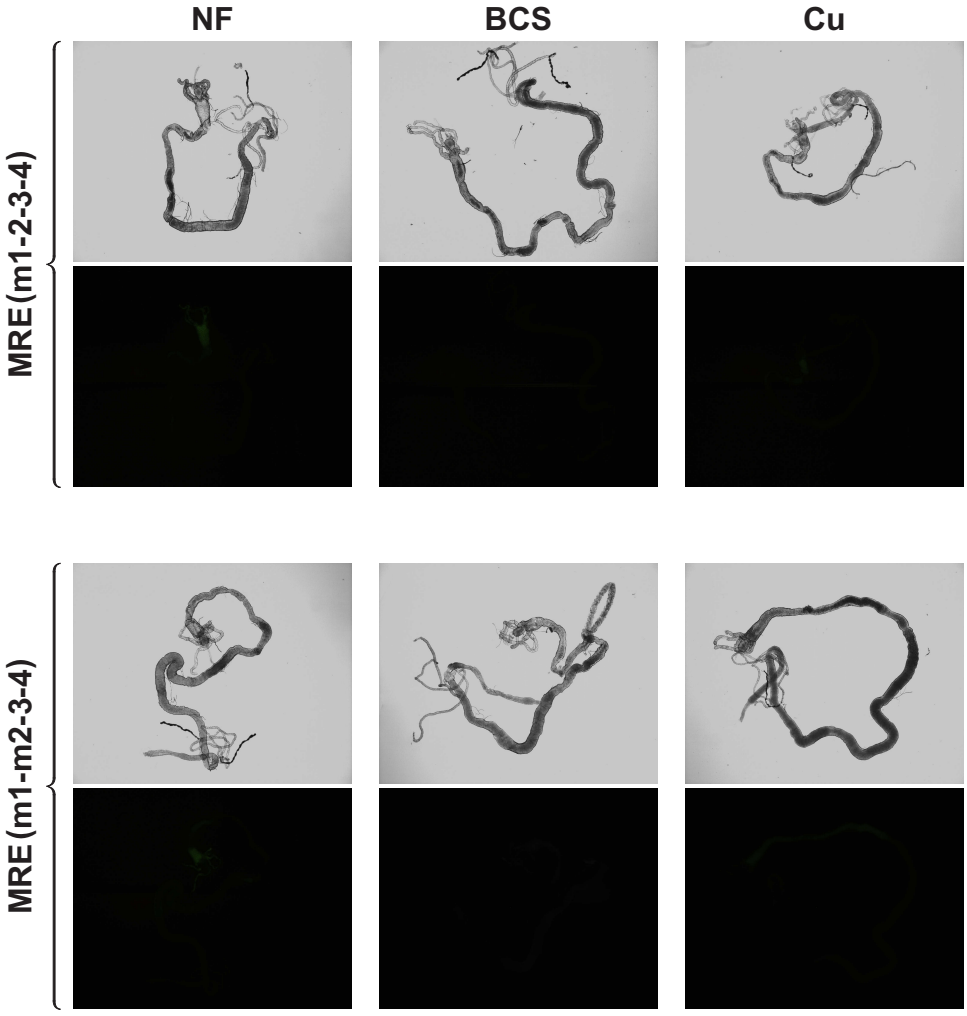
D. melanogaster 1401 ACCTCAGCAGGTCTA---GCTCTTATCACTG---TGCCTC---CCC-GAT
D. yakuba        1398 ACCTCAGCAGGTCTA---GCTCTTATCACTG---TGCTTC---CCC-GAT
D. pseudoobscura1388 CCATGAACAG-TGTT---GCGGTTATCACTAACCTGTCTC---CCGTGAA
D. virilis       1400 AGTTAAGCAAGTGTCAACGCTGTTATCACTCA---GGCTCAGGCACTTAA
consensus        1701    T A CA  T T    GC  TTATCACT     G  TC   C    A

D. melanogaster 1441 CGCCAGTCGGATTTTCCAC-------GCCTTCTAT----CTATATCTATA
D. yakuba        1438 CGCCAGTCGGATTTTCCAC-------GCCTTCTGTTTGACTATATCTGTG
D. pseudoobscura1431 CAGCTTTCGGCTTTTCCCTAATTTGTGCTTTATTTA--ATTATAT---TA
D. virilis       1447 CG---TACG--TTTTCACTTG-----GCGGTTAATTGAATTCTAT----T
consensus        1751 C      CG  TTTTC          GC  T   T     T TAT
                            Translation start of Ctr1B
D. melanogaster 1480 CTACACCAGC---AGC-CCAAG----ATG
D. yakuba        1481 TCA-ATAAGC---AGC-CCAAG----ATG
D. pseudoobscura1476 TCAAATAAACCTGACCGCCAAG----ATG
D. virilis       1483 CAAAAT-----------CCAATTACAATG
consensus        1801   A A            CCAA     ATG
```

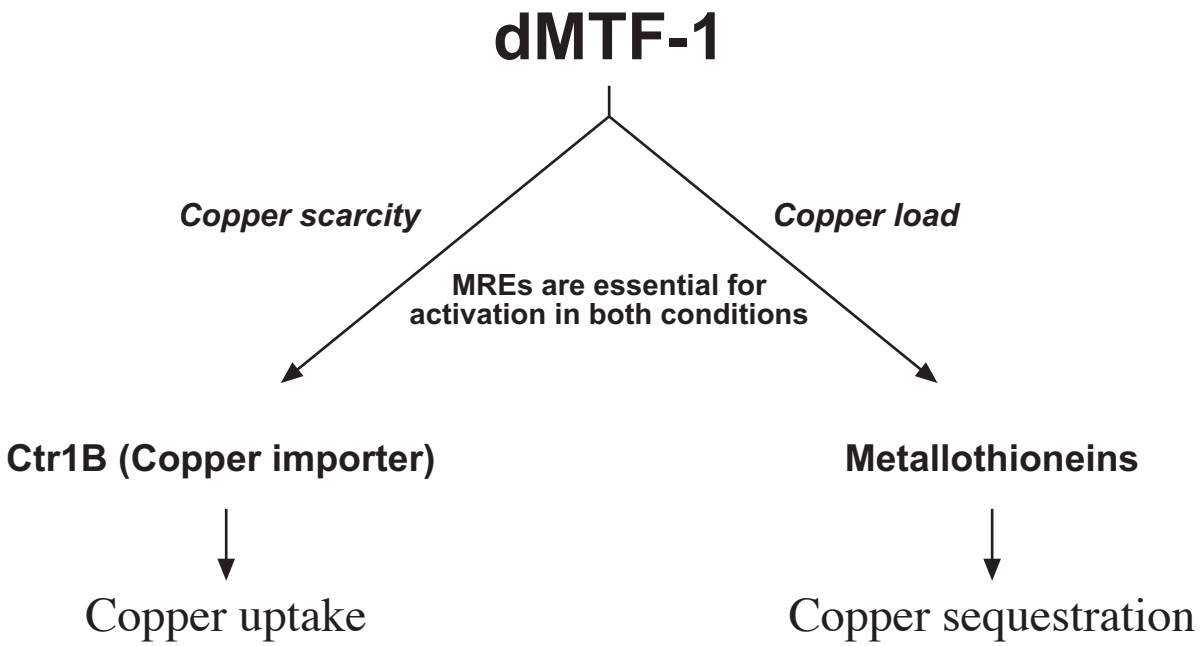
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table 1 Selvaraj et al. MS no: 037630

Constitutive expression of Ctr1B rescues the lethality of the dMTF-1 mutants in copper scarcity

	Genotype	Animals surviving to adult stage (%)			
		NF	BCS 50 μM	BCS 100 μM	BCS 200 μM
a)	$\frac{dMTF\text{-}1^{140\text{-}1R}}{dMTF\text{-}1^{140\text{-}1R}}$	60	0	0	0
b)	$\frac{dMTF\text{-}1^{140\text{-}1R},\ UAS\text{-}Ctr1B}{dMTF\text{-}1^{140\text{-}1R},\ UAS\text{-}Ctr1B}$	60	0	0	0
c)	$\frac{dMTF\text{-}1^{140\text{-}1R},\ actin\text{-}Gal4}{dMTF\text{-}1^{140\text{-}1R},\ UAS\text{-}Ctr1B}$	0	4 (n = 11)	20 (n = 80)	0

Supplementary Materials

DNA constructs

Ctr1B expression vector: The *Ctr1B* coding region was amplified by PCR (with primers 5'-tggactagatctCAAcATGGATCACGGCTCGGAT-3' and 5'-gtcagt-agatctCTAAGGACAGCACTCGCT-3') from a Ctr1B cDNA clone and inserted into SmaI site of pBluescript. The obtained construct was digested with EcoRI & NotI and the fragment was cloned (using EcoRI & NotI) into the P element vector pUAST.

Reporter constructs: The genomic region of *Ctr1B* was amplified by PCR (with primers 5'-CCACCTAAGCAGTTTAGCAAAAGC-3' and 5'-CTAAGGACAGCA-CTCGCTCTCGTC-3') and cloned into the EcoRV site of pBluescript. The obtained construct was used to amplify the regions of interest using the following oligos. 5'-cggaattcCCAGTTAGAAATAGCAC-3' and 5'-gaggatccATCCATCTTG-GGCTGCTG-3' for AH1, 5'-gcgaattcCCACCTAAGCAGTTTAGC-3' and 5'-gagg-atccATCCATCTTGGGCTGCTG-3' for AH2, 5' gcgaattcCCACCTAAGCAGTTT-AGC-3' and 5'-gaggatccAGGACAGCACTCGCTCTC-3' for AH3. EGFP was amplified with primers 5'-gaggatccGTGAGCAAGGGCGAGGAG-3' and 5'-tagcg-gccgcTTACTTGTACAGCTCGT-3'. To generate reporter constructs, a three-way ligation was performed with amplified products and a standard P element vector pCasper. For the MRE mutations the following oligos were used. 5'-gcaatatcgt-taatgtttGACGCACgtcgcccatttttcg-3' for MRE(m1-2-3-4), 5' gcaatatcgttaatgtttGA-CGCACgtcgcccatttttcg-3' and 5'-gcaggaatgtccgtagaatttaGACACACggccgcaga-ag-3' for MRE(m1-m2-3-4), 5'-gcaatatcgttaatgtttGACGCACgtcgcccatttttcg-3', 5'-gcaggaatgtccgtagaatttaGACACACggccgcagaag-3', 5'-cacggccgcagaaggttGAC-GCACggccatcaattttggc-3' and 5'-gataatctgaattgcaataaattcGACACACgaatctcgat-tgtcgaacc-3' (MREm1-m2-m3-m4). All constructs were made using Quick Change Multi Site-Directed Mutagenesis Kit (Roche). The mutations were verified by sequencing.

"Minipromoter": The synthetic "minipromoter" construct containing 4xMtnB MREs was generated by inserting the annealed oligos 5'-CGAGACTCGGTGCACACG-AACTCCTTTTTGCGCACGCGATTAGGCTTGCACACGACGTGAATTTTGCACT CGTTCGG-3' and 5'-TCGACCGAACGAGTGCAAAATTCACGTCGTGTGCAAG-CCTAATCGCGTGCGCAAAAAGGAGTTCGTGTGCACCGAGTCTCGAGCT-3' into SacI, SalI sites of OVEC (Westin et al. 1987; Zhang et al. 2001). The 4x*Ctr1B* MREs and 4x*Ctr1B* MRE (1) reporter constructs were made by introducing oligos 5'-CGAGATGTTTTGCGCACGTCGCCATTTATGCACACG-GCCGCAGGTTTG-CGCACGGCCATAATTCTGCACAC-GAATCG-3' and 5'-TCGACGATTCGTGTGCAGAATTATGGCCGTGCGCAAACCTGCGGCCGTGTG CATAAAAAATGGCGACGTGCGCAAAACATCTCGAGCT-3' for 4x*Ctr1B* MREs and 5'-CGAGTGTTTTGCGCACGTCGCCTGTTTTGCGCACGTCGCCTGTTT-TGCGCACGTCGCCTGTTTTGCGCACGTCGCCG-3' and 5'-TCGACGGC-GACGTGCGCAAAACAGGCGACGTGCGCAAAACAGGCGACGTGCGCAAAA-CAGGCGACGTGCGCAAAACACTCGAGCT-3' for 4x*Ctr1B* MRE (1), also into SacI, SalI sites of OVEC.

Ctr1B upstream region from D. virilis: The primers 5'-GTGTCGTAGACAATGGT-CCA-3' and 5'-AGCCAGTAGTTGAAGGTCATGAA-3' were chosen from the *D. melanogaster CG9613* (upstream gene of *Ctr1B*) coding region and *D. melanogaster Ctr1B* coding region, respectively. This primer pair amplified a single 3.5 kb fragment from *D. virilis* genomic DNA. The amplified product from *D. virilis* was cloned into the EcoRV site of pBluescript and sequenced.

Fly stocks and genetics

All the P element vectors were injected into *y w* embryos and at least two independent insertions of each transgene were analyzed in all the experiments.

The following fly stocks were used in this study:

y w; dMTF-1$^{140\text{-}1R}$/dMTF-1$^{140\text{-}1R}$ (dMTF-1 null mutant*)*

y w; Ctr1B$^{3\text{-}4}$/Ctr1B$^{3\text{-}4}$ (Ctr1B null mutant*)*

y w; dMTF-1$^{140\text{-}1R}$, actin-Gal4/TM6B

The following fly stocks were generated:

y w; UAS-Ctr1B UAS-Ctr1B

y w; dMTF-1$^{140\text{-}1R}$,UAS-Ctr1B/dMTF-1$^{140\text{-}1R}$,UAS-Ctr1B

y w; AH1/AH1

y w; AH2/AH2

y w; AH3/AH3

y w; AH2/AH2; dMTF-1$^{140\text{-}1R}$/dMTF-1$^{140\text{-}1R}$

y w; AH3/AH3; dMTF-1$^{140\text{-}1R}$/dMTF-1$^{140\text{-}1R}$

y w; MRE(m1-m2-m3-m4) MRE(m1-m2-m3-m4)

y w; MRE(m1-m2-3-4)/MRE(m1-m2-3-4)

y w; MRE(m1-2-3-4)/MRE(m1-2-3-4)

y w; AH3/AH3; Ctr1B$^{3\text{-}4}$/Ctr1B$^{3\text{-}4}$

y w; poxMRD/poxMRD; Ctr1B$^{3\text{-}4}$/Ctr1B$^{3\text{-}4}$

y w; MRE(m1-m2-m3-m4)/MRE(m1-m2-m3-m4); *Ctr1B$^{3\text{-}4}$/Ctr1B$^{3\text{-}4}$*

y w; MRE(m1-m2-3-4)/MRE(m1-m2-3-4); Ctr1B$^{3\text{-}4}$/Ctr1B$^{3\text{-}4}$

EMSA

The following MRE oligonucleotides were used for EMSA:

MRE-s (consensus MRE sequence, positive control):

5'-CGAGGGAGCTCTGCACACGGCCCGAAAAGTG-3' and

3'-TCGAGCTCCCTCGAGACGTGTGCCGGGCTTTTCACAGCT-5'.

MRE1 (Ctr1B):

5'-TCGTTAATGTTTTGCGCACGTCGCCCAT-3' and

3'-AAAATGGGCGACGTGCGCAAAACATTAA-5'

MRE [2+3] (Ctr1B):

5’-GAATTTATGCACACGGCCGCAGAAGGTTTGCGCACGGCCA-3' and

3'-TGATGGCCGTGCGCAAACCTTCTGCGGCCGTGTGCATAAA -5'

A family knockout of all four *Drosophila* metallothioneins reveals a central role in copper homeostasis and detoxification

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Key words: metallothionein, transcription, copper, gene knock-in, gene knockout, MRE, lifespan

Abstract

Metallothioneins are ubiquitous, small, cysteine-rich proteins with the ability to bind heavy metals. In spite of their biochemical characterization their *in vivo* function remained elusive. Here we report the generation of a metallothionein gene family knockout in *Drosophila* by targeted disruption of all four genes (*MtnA-D*). These flies are viable if raised in standard laboratory food. During development however, they are highly sensitive to copper, cadmium, and to a lesser extent to zinc load. Metallothionein expression is particularly important for male viability; while copper load during development affects males and females equally, adult males lacking metallothioneins display a severely reduced life span, possibly due to copper-mediated oxidative stress. Using various reporter gene constructs, we find that different metallothioneins are expressed with virtually the same tissue specificity in larvae, especially in the intestinal tract at sites of metal accumulation, including the midgut's "copper cells". The same expression pattern is observed with a synthetic minipromoter consisting of four tandem metal response elements (MREs) only. From these and other experiments we conclude that tissue-specificity of metallothionein expression is a consequence, rather than a cause of metal distribution in the organism. The bright orange luminescence of copper accumulated in copper cells of the larval midgut is severely reduced in the metallothionein gene family knockout as well as in mutants of the metal-responsive transcription factor (MTF-1), the main regulator of metallothionein expression. This indicates that an *in vivo* metallothionein-copper complex forms the basis of this luminescence. Strikingly, metallothionein mutants show an increased, MTF-1 dependent activation of metallothionein promoters in response to copper, cadmium, silver, zinc and mercury. We conclude that free, but not metallothionein-bound metal, triggers the activation of MTF-1, and that metallothioneins regulate their own expression by a negative feedback loop.

Introduction

Copper is an essential trace element with an important role in several cellular processes such as respiration, oxidative stress defense, immune function, and angiogenesis (22, 45, 62). Copper is found in a number of important enzymes and proteins, such as cytochrome c oxidase, Cu,Zn-superoxide dismutase (SOD1), tyrosinase, lysyl oxidase, multicopper oxidase, APP and PrP. In many of the copper-dependent enzymes, copper undergoes a change in redox state, an ability which is both essential and potentially harmful to the cell, as uncontrolled redox cycling of copper can generate reactive oxygen species (ROS). Indirect damage by copper to the cell through ROS affects lipid membranes, DNA and proteins (19). In addition to the generation of ROS, copper may manifest its toxicity by displacing other metal cofactors from their natural ligands (48). Several cellular systems that are largely conserved in eukaryotes ensures sufficient uptake of copper and control its action to minimize damage (reviewed in (2, 49). In metazoans, the copper importer Ctr1 mediates uptake, while the copper exporter ATP7 mediates export of excess copper, and copper chaperones deliver copper selectively to copper-dependent enzymes (10, 23). Cells protect themselves from toxic effects of copper and other transition metals of the group Ib and IIb, hereafter referred to as heavy metals, by sequestering them in metallothioneins. This way the cell keeps the free metal concentration extremely low (44, 51). Metallothioneins are small proteins with high cysteine content and the ability to bind up to 9 copper ions or other metal ions, such as zinc and cadmium (reviewed in (29)). Metallothioneins are found from yeast to humans and even in some plants and prokaryotes. Their conservation and the presence of multiple gene copies imply an important cellular function, notably in metal homeostasis and defense against toxicity of heavy metals. Yeast cells have two metallothioneins that confer copper resistance (12, 27). Knockout mice for two of the four metallothioneins, *Mt1* and *Mt2*, are viable but sensitive to cadmium and zinc loads (31, 39, 42). In cell culture and in flies, metallothionein gene duplications correlate with increased resistance to copper (13, 16, 38). Metallothioneins are also involved in the defense against oxidative stress. They react *in vitro* with ROS, and *in vivo* they are able to reduce ROS-mediated DNA strand break formation and lipid peroxidation (1, 30, 50, 61, 63).

Transcription of metallothioneins in mammals is induced upon metal load and oxidative stress via the metal-responsive transcription factor MTF-1, which binds to MREs (metal response elements) in the promoter region. Cultured MTF-1 knockout cells show higher susceptibility to hydrogen peroxide and cadmium (20). Previously, we have reported the characterization of MTF-1 mutant flies. They are viable but highly sensitive to copper, zinc and cadmium load, as well as to copper depletion (17). Similar to the situation in mice, basal and heavy metal induced transcription of the four *Drosophila* metallothionein genes (*MtnA, MtnB, MtnC* and *MtnD*) depends on MTF-1 (17, 25). Despite considerable progress in our understanding of the transcriptional response to heavy metals, the regulation of MTF-1 activity is not well understood. What is known is that one or more of the six zinc fingers of MTF-1 have a relatively low affinity for zinc. Apothioneins, which are newly synthesized thioneins that are not conjugated to metals, can effectively compete with MTF-1 for zinc binding which inhibits MTF-1 DNA binding and transcriptional activation in a cell-free transcription system (7, 25, 69). Unlike zinc, other heavy metals such as copper or cadmium, cannot directly stimulate MTF-1 DNA binding activity in a cell free extract. These *in vitro* experiments have led to the model that zinc binds and activates MTF-1 directly, while cadmium or copper, which have a higher affinity to metallothioneins than zinc, displace the latter from metallothioneins, and subsequent binding of the released zinc to MTF-1 leads to its activation (69). In mammals, MTF-1 resides largely in the cytoplasm, from where it translocates to the nucleus upon cell stress, notably metal load. Activation and nuclear translocation of MTF-1 is rapid and transcript levels peak after about 6 hours in the mouse liver and 24 hours in *Drosophila* cultured cells (8, 32, 43, 55, 59). Besides signals leading to the activation of MTF-1, negative regulators of MTF-1 also exist, however the nature of this regulation has not been identified. Newly synthesized apo-thioneins are good candidates for negative feedback of zinc-mediated induction, while zinc-loaded metallothioneins may contribute to the indirect induction by cadmium or copper.

Here we report the generation of a gene family knockout of all four *Drosophila* metallothioneins by means of homologous recombination. Since the four genes are interspersed with other genes, they had to be selectively mutated one-by-one. This represents the first example of a knockout of all metallothioneins in a higher eukaryote.

The resulting flies are viable and fertile, but sensitive to elevated concentrations of copper or cadmium, but not to copper depletion. We also report both a negative and a positive effect of copper on lifespan. Mild copper load shortens lifespan of metallothionein mutant males and to a lesser extent also of females, perhaps due to increased copper-mediated oxidative stress. On the other hand, an adequate copper supply is necessary to allow for a normal life span, possibly by ensuring proper functioning of the antioxidant enzyme Cu,Zn-SOD. Metallothioneins are expressed in a tissue-specific manner especially at sites of metal accumulation. Binding of copper to metallothioneins leads to an orange luminescence in copper-accumulating cells of the midgut, the so-called copper-cells. Once heavy metal is bound in such a manner it no longer triggers the activation of metallothionein genes, which generates a negative feedback on metallothionein gene expression.

Materials & Methods

pTARG, a versatile vector for gene targeting and *Drosophila* transgenesis

The plasmid pTARG is a P-element vector derived from pCasper4. It includes a multiple cloning site, an *I-Cre*I recognition sequence, a *white* cDNA driven by the artificial promoter *3xP3* (6, 26), two *loxP* sites and two FRT sites to release an episome for gene targeting. The 3xP3-*white* gene serves as a marker for transgenesis and for gene targeting and the loxP sites can serve to eliminate the white marker gene. Targeting is performed as described previously (17, 53). Flies carrying the *3xP3-white* marker gene show a typical orange eye color with a well-visible pseudopupilla and red pigment in the ocelli. This phenotype shows almost no variation between different transgenic lines and can easily be distinguished from a miniwhite transgene. Plasmid DNA and the entire sequence information are available upon request.

Generation of a metallothionein gene family knockout / knock-in

To generate metallothionein mutations, we used the method of ends-in targeting (53). Gene targeting events were selected based on the resistance of the *white*$^+$ (w^+) marker gene to ey-Flp, based on the presence of the mutation replacing the ATG with a *Not*I site, and based on the ability to undergo efficient elimination of the w^+ marker by the endonuclease *I-Cre*I, indicating the existence of a gene-duplication that undergoes recombination and reduction to a single copy leading to the loss of the w^+ marker. Final evidence of gene targeting was obtained after reduction to a single copy, breeding of this reduction event to homozygosity and analysis by PCR using primers flanking the metallothionein gene. This PCR amplifies both wild type and mutant DNA. *Not*I digestion or sequencing of the coding region using a nested primer revealed whether the mutation was present in homozygous form. The molecular nature of each mutation is given in Supplementary Table 1 and primer sequences are given in Supplementary Materials. All four metallothioneins were targeted in the background of a third chromosome derived from OregonR that contains an *MtnD* gene with a premature stop

codon (allele $MtnD^{*}$) (17). Different stocks are therefore isogenic for the third chromosome. Mutant alleles were designated $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnC^{\Delta ATG}$ and $MtnD^{\Delta ATG}$. In addition to the $MtnD^{\Delta ATG}$ targeted allele, a knock-in allele of *MtnD* was generated by introducing a dsRed gene including an SV40-pA terminus into the same *Not*I site that replaced the ATG of *MtnD*. This heterologous insertion did not obviously affect the efficiency of ends-in gene targeting. Targeting and verification of the targeted events was performed as with other ends-in constructs. PCR using two primers flanking the region of interest showed that in homozygous knock-in flies only a band of about 1.3 kb longer than the wild type band can be detected (Fig. 1B, C). This allele was designated $MtnD^{dsRed}$. To test whether metal load could influence targeting efficiency, we added copper to the food. However targeting efficiency at the *MtnC* locus did not increase. Construction of a quadruple metallothionein (*qMtn*) mutant was done in subsequent steps of targeting: $MtnA^{\Delta ATG}$, $MtnD^{*}$ and $MtnB^{\Delta ATG}$, $MtnD^{*}$ were recombined to yield $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnD^{*}$, also termed *tMtn**. Targeting of MtnC was performed in the *tMtn** mutant background, resulting in $MtnA^{\Delta ATG}$, $MtnC^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnD^{*}$, or *qMtn**. The genotype before the reduction, that has the *MtnC* gene marked with w^{+} ($MtnA^{\Delta ATG}$, $MtnC^{w+}$, $MtnB^{\Delta ATG}$, $MtnD^{*}$) was used to select for a meiotic crossing over between $MtnC^{w+}$ and $MtnD^{dsRed}$. Flies with a chromosome that is both w^{+} and $dsRed^{+}$ may also carry the mutant $MtnB^{\Delta ATG}$ allele if the crossover occurred within the very short (31.5 kb) interval between *MtnB* and *MtnD*. We recovered a single crossover event of this kind among approx. 5000 chromosomes screened. The allele $MtnC^{w+}$ is not a null allele, since it is a duplication consisting of a wild type and a mutant copy of *MtnC*, flanking the w^{+} gene. Reduction of this duplication to single copy finally resulted in the genotype $MtnA^{\Delta ATG}$, $MtnC^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnD^{dsRed}$, also termed $qMtn^{dsRed}$. For rapid genotyping, primers were designed that specifically recognize the mutant alleles.

Metallothionein rescue experiments

For the MtnB genomic rescue construct, the region from 1529 bp upstream of the ATG start codon to 687 bp downstream of the stop codon was cloned into the widely used pCasper4 vector, and for the MtnA genomic rescue construct the region from 569 bp

upstream of the ATG start codon to 227 bp downstream of the stop codon was used. These constructs contain a cluster of upstream metal-response elements (MREs), to which MTF-1 binds to activate transcription. Constructs were injected in *qMtn** mutant flies to ensure comparison between isogenic flies. For rescue analysis, *qMtn** mutant females were crossed with males of the same genotype, but carrying the w^+ marked transgene in a heterozygous state.

Fluorescent protein reporter

For the construction of the MtnB-EYFP reporter, the segment -1460 to +50 relative to the transcription start point was chosen and cloned in front of EYFP in a pCasper4 backbone. Cloning of the MtnA-EYFP reporter construct was reported previously (3). For the construction of 4xMRE-EYFP, the sequences derived from the MtnB or the Ctr1B promoter were chosen as described previously (56, 68). Transgenics were produced by P-element mediated transgenesis. For analysis of EYFP expression by microscopy, flies were allowed to deposit eggs in the food and were raised until third instar larvae. Guts were dissected and mounted in glycerol. The construction of a Ctr1B-EGFP reporter transgene was reported previously (56).

Fly food

1 liter of fly food was composed of 55g corn, 100g yeast, 75g sugar (glucose), 8g agar, 15ml Nipagin and 10g wheat. For longevity and toxicity experiments, food was supplemented with $CdCl_2$, $CuSO_4$, $ZnCl_2$, $HgCl_2$, $AgNO_3$ or BCS disodium salt hydrate (Sigma-Aldrich 14,662-5) to the concentrations indicated. BCS is a specific copper chelator that is used to deplete the food of copper.

Toxicity experiments

Flies were allowed to deposit a determined number of eggs on food and eclosing adults were counted. Survival on metal-supplemented food was normalized to survival of the corresponding strain on normal food. Survival from egg to adult on food without metal

supplement varies between 50 and 70%. Experiments were performed at least 3 times and also at different concentrations of the same metal. An extensive analysis of the sensitivity of a wild type strain (*y w*) and the *MtnD** mutant strain to various concentrations of Zn, Cu, Cd, Hg and the copper-specific chelator BCS (Sigma-Aldrich 14,662-5) is presented in supplementary Fig. 2. Flies were kept at the standard temperature of 25°C.

Quantification of metallothionein transcripts

Larvae were either continuously raised on the indicated type of food, or transferred for 6 hours either to normal food or to metal-supplemented food. Only third instar feeding larvae were used for the analysis. Total RNA was extracted using the TRIzol reagent (Life Technologies). Nuclease S1 mapping of transcripts with 50 μg of total RNA was performed as described previously (65). The gels were developed using PhosphorImager (Molecular Dynamics) and bands were quantified. The signal from the endogenous actin5c gene was used for normalization of metallothionein transcript levels.

Life span experiments and SOD1 measurements

Life span experiments were carried out at 28°C. Isogenic flies were grown from embryonic stage on food with or without metal supplement. To ensure that the results are comparable, special care was taken to grow heterozygous and homozygous mutant flies on the very same batch of food and even in the same tube. This was achieved by crossing homozygous *qMtn** mutant virgins with heterozygous mutant males, resulting in 50% homozygous and 50% heterozygous mutant offspring from the same vials. This was necessary because the complex composition of fly food can lead to variation in the metal content of flies grown on different preparations of the same ingredients (unpublished data). For life span analysis, males or virgin females were kept in groups of 30 and transferred twice a week to new tubes with or without metal supplement and dead flies were counted every day. For each condition, 80 to 400 flies were analyzed. For Fig. 6C, flies were raised on the indicated type of food, but all were kept as adults on normal food, unlike the experiments in Fig. 6A,B, and D, where they were kept on the same food on

which they were raised. SOD1 activity was measured using the Calbiochem superoxide dismutase assay kit Cat. No. 574600. For total protein extracts, about 30 flies were homogenized in a buffer consisting of 50mM Tris (pH 8.0), 0.125mM NaCl, and the protein content was quantified using the Bradford assay from Biorad.

Imaging and Microscopy

Copper cell luminescence and fluorescent protein expression in dissected guts was analyzed with a Leica DRB fluorescence stereomicroscope equipped with a Zeiss Axiocam. Confocal images were taken with a Leica SP1 UV CLSM.

Metal measurements

Groups of about 15 adult males (2-3 days old) were weighed and analyzed by ICP-MS (*inductively-coupled plasma mass spectrometry*), as described previously (57). Flies were dissolved in 65% HNO_3 in a microwave oven and then diluted to a 6.5% HNO_3 solution for analysis. ICP-MS was performed using a HP4500 Series 300 ShieldTorch System instrument (Agilent, Waldbronn, Germany) in peak-hopping mode with spacing at 0.05 amu, 3 points/peak, 5 scans/replicate, 2-3 replicates/sample, and an integration time of 400 ms/point. The rate of plasma flow was 15 L/min with an auxiliary flow of 1.0 L/min. The RF power was 1.2 kW. The sample was introduced using a cross-flow nebulizer at a flow rate of 1.06 L/min. The apparatus was calibrated using a 6.5% HNO3 solution containing Cu at 5, 10, 25, 50, and 100 ppb with ^{103}Rh, the internal standard for all isotopes of Cu.

Results

A metallothionein gene family knockout

The *Drosophila* genome harbors four metallothionein (*Mtn*) genes, designated *MtnA-D* (17). They encode short proteins of 40-44 aa which contain 10-12 cysteine residues in configurations CysXCys and CysXXCys that are characteristic for metallothioneins (52). MtnB, MtnC and MtnD display more than 67% aa identity and probably arose by gene duplications, whereas MtnA only shares the cysteine motifs with the other three metallothioneins. These relations are also reflected in the genomic organization. All four genes are located on chromosome 3, but while *MtnA* localizes to cytological position 85E, *MtnB*, *MtnC* and *MtnD* all localize to 92E within a segment of 170kb. The 170kb segment however also harbors 11 genes interspersed with the metallothioneins (Fig.1A). The small size of the metallothionein genes as well as their genomic organization precluded a comprehensive analysis by classical genetic methods or by other more recent methods based on random introduction of mutations. Therefore, we employed the recently introduced tool of gene targeting by homologous recombination in *Drosophila* to generate flies mutant for single metallothioneins and for combinations of them, including a mutant for all four metallothioneins, in the same genetic background derived from OregonR (53). This fly strain carries a spontaneous truncation allele of *MtnD,* hereafter referred to as *MtnD** (17). The mutations were introduced by ends-in gene targeting and designed such that a *Not*I recognition site replaced the ATG start codon and several neighboring amino acids, resulting in the alleles $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnC^{\Delta ATG}$, *and* $MtnD^{\Delta ATG}$. Even though promoter sequences are still intact and lead to the production of mutant mRNAs, these cannot be translated and at least some of them are unstable compared to wild type, as revealed by a severe reduction of full-length mRNA levels (supplementary Fig.1). The introduced mutations therefore most likely represent null mutations. We also generated a knock-in replacement allele of MtnD, designated $MtnD^{dsRed}$, resulting in expression of a red fluorescent dsRed protein under the control of the endogenous *MtnD* promoter (Fig.1B, C). This allele is both a null mutation and a reporter gene for the expression of the *MtnD* locus. In contrast to a reporter transgene, such a knock-in reporter is not subject to position effects that might affect the pattern and

strength of expression.

Drosophila metallothionein mutants are highly sensitive to copper and cadmium, to a lesser extent to zinc, but not to mercury or silver load, or to copper depletion

Flies mutant for all four metallothioneins, $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnC^{\Delta ATG}$, $MtnD^{*}$ or $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnC^{\Delta ATG}$, $MtnD^{dsRed}$, hereafter referred to as *quadruple Mtn* (*qMtn* and* $qMtn^{dsRed}$, respectively) mutant flies, are viable and fertile, but highly sensitive to copper or cadmium load in the food (Fig.2B). Whereas 0.5 mM copper does not affect the survival of wild type flies, *qMtn* or *tMtn* ($MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnD^{*}$) mutant flies are unable to survive when raised on this food apart from a few escapers that die shortly after eclosion. Death can occur at different stages of development, but high concentrations inevitably result in early larval death. Lower concentrations allow the progression to later larval stages or even to adulthood. Similarly to copper, 50 μM cadmium is lethal for *qMtn* mutants, but not for wild type flies. The protective effect of metallothioneins correlates with the transcriptional activation of metallothionein promoters and with the metal binding ability of *Drosophila* metallothioneins. Previous work has demonstrated that both copper and cadmium are very strong inducers of all four metallothioneins and that both MtnA and MtnB have a high capacity to bind to copper and cadmium ions (14, 17, 64, 68). *qMtn* mutants are as sensitive to copper or cadmium as a deletion mutant of *MTF-1,* thus providing a clearcut explanation for the metal sensitivity of *MTF-1* mutants in which transcription from the *Mtn* loci is abrogated. Unlike *MTF-1* mutants, *qMtn* mutants are not sensitive to copper depletion. This is not unexpected since MTF-1 also controls copper import by the activation of the gene for the copper importer Ctr1B (56). Consistent with this, under conditions of copper scarcity or copper load *tMtn* mutants maintain a body copper level similar to wild type (Fig. 2D). These findings rule out any essential role of metallothioneins in copper-import or as copper-chaperones for the transfer of copper to copper-dependent enzymes (see also (34)). Surprisingly, even though *MTF-1* mutants are clearly more sensitive to zinc than wild type flies, zinc sensitivity in metallothionein mutants was only marginally elevated (Fig.2B). *MTF-1* mutants, but not *qMtn* mutant flies show a clearly elevated sensitivity also to Hg and Ag.

The analysis of single metallothionein mutants or of different combinations

revealed that MtnB plays a major role in the defense against the toxicity of cadmium, whereas MtnA is of major importance under copper load (Fig.2B). Accordingly, double mutants of *MtnA* and *MtnB* show enhanced sensitivity to both metals. The role of MtnC and MtnD in the defense against heavy metals is less prominent. $MtnD^{*}$ alone or the double mutant $MtnC^{\Delta ATG}$, $MtnD^{*}$ are only marginally more sensitive to either cadmium or copper in comparison to wild type. The role of MtnC and MtnD becomes only obvious in the absence of both MtnA and MtnB: thus, the most sensitive genotypes of all are *qMtn** and $qMtn^{dsRed}$ (Fig.2B, D). Differences between individual metallothioneins were also revealed by rescue analysis. Both genomic transgenes of MtnA tested and to a lesser extent a transgene of MtnB were able to partially rescue copper sensitivity of a *qMtn** mutant fly, consistent with the major role of MtnA in defence against copper toxicity (Fig.2C).

Metallothioneins not only play an important role during a long-term exposure to copper, but also in response to acute copper exposure (Fig. 2C). We transferred third instar larvae from either copper depleted or normal food into food with elevated copper concentration. The effect was most striking in a transition from copper starvation to copper abundance. While wild type larvae survived this transition well, metallothionein mutants died after several hours, without developing into pupae. This experiment shows that metallothioneins are important to cope with copper fluctuations. In wild type larvae, basal level expression together with a rapid induction of metallothionein genes is able to protect the organism from a sudden increase in copper concentration.

Metal-response elements are sufficient to mediate tissue-specific expression of metallothioneins

Metallothioneins are expressed in a tissue-specific manner in virtually identical expression patterns (Fig.3, supplementary Fig.3). In larvae, the sites of expression include the so-called copper cells, or cuprophilic cells, copper accumulating cells in the midgut. Mtn expression can also be observed at the midgut constriction, in malpighian tubules, in the salivary glands, the fat body, the cuticle and in trachea, but not in the brain or in imaginal discs, even in response to a very high metal load (see also (15)). Copper,

cadmium or zinc preferably induce Mtn expression in different regions of the gut: copper induces mostly in the copper cells, while zinc and cadmium induce at the midgut constriction and in the posterior midgut. To investigate the reason for this tissue-specific induction, we analyzed the expression pattern of EGFP under the control of the synthetic 4xMRE promoter with metal-response elements (MREs) derived from either *MtnB* or *Ctr1B*. Ctr1B is expressed in an MTF-1 dependent manner in the midgut, in a pattern differing from metallothioneins, but a minimal synthetic promoter consisting merely of the MREs of this gene is inducible by metal load (56). Both synthetic promoters show a striking overlap of EGFP with dsRed expressed from the $MtnD^{dsRed}$ knockin allele, which also is similar to the expression pattern produced by the full-length MtnB promoter. Not only basal expression, but also metal-induced expression of 4xMRE where the MREs are derived from MtnB occurs in the very same tissues as for MtnB itself (supplementary Fig. 3B). Even a ubiquitous overexpression of MTF-1 under the control of a tubulin promoter does not alter the expression pattern of the allele $MtnD^{dsRed}$ (not shown).

Metallothioneins form a luminescent complex with copper

Specific cells in the *Drosophila* midgut, known as copper cells, show a bright orange luminescence under UV light when larvae are fed with copper (47). Cu(I) thiolates as well as Cu-metallothionein complexes in yeast and *Neurospora crassa* are known to yield a characteristic orange light emission when excited with ultraviolet light (5). This luminescence depends on a Cu(I) state, and occurs only when the complex is shielded from solvent quenching (9, 35). It was already proposed that *Drosophila* copper cell luminescence is due to a copper-metallothionein complex, as expression of metallothioneins and luminescence coincide (Fig.3B and (41)). In order to test this hypothesis, we examined the guts of *MTF-1* and *qMtn* mutant larvae fed with copper. Indeed, copper cell luminescence was strongly reduced, in either *qMtn* or *MTF-1* mutants, but not completely absent (Fig.4). As the expression of metallothioneins in copper cells depends on the presence of copper, the $MtnD^{dsRed}$ knockin allele served as an important control. We observed a strong and specific induction of $MtnD^{dsRed}$ in copper cells of both mutant and wild type larvae, showing that the lack of metallothioneins does

not affect the morphology of copper cells nor lead to a reduced copper import. A complex of metallothionein with copper therefore accounts for the majority of copper cell luminescence and a loss of metallothioneins results in an increased solvent-accessibility of copper. However, in *qMtn* as well as in *MTF-1* mutants a residual luminescence remains, possibly due to copper binding to other cellular components which are less able to shield copper from water (Fig. 4 and not shown). Interestingly, expression of the dsRed knock-in reporter at the *MtnD* locus was much stronger in the homozygous *qMtn* mutants than in heterozygous controls (Fig. 4). This prompted us to test a possible autoregulation of metallothionein expression.

Metallothioneins negatively regulate MTF-1 transcriptional activity *in vivo*

We tested the activity of the endogenous *MtnC* promoter as well as the expression levels of an MtnB-EYFP reporter gene in both the wild type and in metallothionein mutants, the latter being represented by *qMtn** or the triple mutant *tMtn** (MtnA, MtnB and MtnD mutated). In *qMtn* and *tMtn* mutants, both promoters show a significantly higher expression level, about 2-3 fold higher than wild type, in response to either a short metal-stimulus (6 h) or to constant metal-exposure (Fig.5A&B). In contrast, the expression of the *Ctr1B* copper importer gene, which also depends on MTF-1 activity (56), is not changed in *qMtn* mutants, as analyzed by a Ctr1B-EGFP reporter signal (Fig.5C).This is consistent with the fact that metallothionein mutants do not differ from wild type in their overall metal content (Fig.2E). This shows that the negative feedback is restricted to metallothionein promoters.

A possible consequence of the negative feedback of metallothionein expression on MTF-1 activity might be that transcription at the metallothionein loci does not only depend on the absolute metal concentrations, but also on the change of metal concentration over time. Consistent with such a scenario, transfer of wild type larvae from normal conditions to food with 50 µM copper, i.e. corresponding to a mild copper load, induces metallothionein transcripts to higher levels than permanent exposure to 50 µM copper (Fig.5 D, E). The lower expression level upon constant copper load probably reflects an already elevated concentration of metallothioneins that are able to chelate free

metal, thus alleviating the need for MTF-1 activation.

Copper shows a dual effect on life span

We also tested the lifespan of *qMtn* mutant and heterozygous wild type flies at different copper levels (Fig. 6A,B). The *Drosophila* cultures were grown throughout development on normal food or food containing 50 µM, 75 µM or 1mM copper, or 500 µM of the specific copper chelator BCS. These concentrations were chosen because they do not affect the survival during development, prior to the life span analysis. 75 µM Cu is not at all toxic for (heterozygous) wild type *Drosophila* during development, and also the survival of *qMtn* mutant males and females is not reduced (not shown). 1mM is however lethal for *qMtn* mutants, but does not affect the viability of (heterozygous) wild type flies during development (not shown). We had determined the total body copper content under these conditions: flies grown at 500 µM BCS, NF or 50 µM Cu contain about 1 ng, 5 ng and 20 ng of copper per mg tissue, respectively (Yepiskoposyan et al., in preparation). A sufficient copper supply is critical for the defense against oxidative stress, since superoxide dismutase (SOD1) is a copper-dependent enzyme (40). While mild copper depletion (100 µM BCS) does not reduce the lifespan of wild type flies, copper starvation in 500µM BCS dramatically reduces life span (Fig.6D). Indeed, larvae grown in 500 µM BCS show very low activity of SOD1 (Fig.6E). *SOD1* mutant flies have previously been shown to have a severely reduced life span (46). Low levels of organismal copper may therefore lead to increased oxidative stress and thereby to shorter life span. On the other hand a mild copper load ~~of~~ (50 µM) does not detectably affect the life span of heterozygous wild type flies, while an increase to 1mM copper slightly reduces life span (8 versus 3 days for males and females, respectively) (Fig. 6C). In normal food, life span is very similar for homozygous *qMtn* mutant flies and heterozygous control flies. However a difference becomes evident with a mild copper load: the mean life span of metallothionein mutant males is 6 days shorter at 50 µM copper and 14 days shorter at 75 µM copper in comparison to metallothionein containing (*qMtn*/+) males. The lack of metallothioneins affects females to a lesser extent. The mean life span of metallothionein mutant females is 3 days shorter at both 50 µM and 75 µM copper than that of

metallothionein containing females. In agreement with a greater dependence of males on functional metallothioneins, males express almost two-fold higher levels of metallothionein mRNAs, even though both sexes contain the same amount of zinc and copper (Fig.6 F, G).

Discussion

The genomic arrangement of the metallothionein genes made the generation of a quadruple knockout a difficult endeavor. All four genes are located on the same chromosome, but they are interspersed with many other genes, which precluded the removal of a large segment. Rather, the genes had to be mutated one-by-one by means of targeted gene disruption. We show here that *Drosophila* metallothioneins have an important role in copper homeostasis as well as in the detoxification of cadmium. While cadmium toxicity results in many cases from industrial pollution and may be regarded as a recent problem, copper homeostasis is physiologically important. The copper tolerance of wild type flies is truly remarkable. In metallothionein mutants, a slight increase of copper levels, whether from a constant exposure or a transient copper shock, results either in larval death or in a shortened lifespan. With the increased sensitivity to copper and cadmium, and to a lesser extent to zinc, metallothionein mutants mirror most but not all aspects of the *Drosophila MTF-1* mutant phenotype. Flies lacking MTF-1 are more sensitive to zinc, silver and mercury than metallothionein mutants. This difference can best be explained by MTF-1 dependent activation of genes involved in metal homeostasis other than the metallothioneins. For example, MTF-1 induces the expression of a putative zinc exporter (CG3994) under conditions of zinc excess, preventing zinc and possibly mercury overload of the cell (Yepiskoposyan et al., in preparation). The sensitivity of *MTF-1* mutants to silver might be due to low expression levels of the copper importer Ctr1B, another MTF-1 target gene (56). Silver exerts its toxicity by competing with copper, and competition is expected to be more severe in *MTF-1* mutants that have lower copper levels than wild type flies (H. Yepiskoposyan & K. Balamarugan, unpublished results). Besides copper and cadmium, mercury, silver and also zinc induce metallothionein transcription (Fig.5A), but these latter metals are equally toxic to wild type and metallothionein mutants. Apparently metallothioneins do not protect against all compounds which induce their synthesis (see also (16)).

Wild type flies can develop at a copper concentration at least 200-fold higher (1 mM) than the normal copper content in the food (5 μM) without affecting survival to adulthood (17). Indeed for most organisms, copper is a relatively benign trace element

thanks to sophisticated transport and detoxification systems. It can however be toxic under special circumstances, such as in association with genetic defects of copper homeostasis or upon environmental accumulation, notably in vineyards where it is used as an antifungal agent. Metallothioneins protect the cells from toxic effects of copper by binding and sequestering the metal inside the protein. This metallothionein-copper complex can be conveniently observed *in vivo* as an orange copper luminescence. The reduction of copper cell luminescence in *qMtn* mutants suggests that copper is now solvent accessible and able to damage the cell, possibly via the generation of ROS and/or ectopic binding to protein sulfhydryl groups. Formation of ROS by copper is often proposed as the major mechanism of copper toxicity (reviewed in (19)). The sensitivity of *SOD1* mutant flies to high levels of copper is in agreement with such a scenario (46). Also in the LEC rat, a model system for a human copper homeostasis disorder, Wilson's disease, copper accumulation in the liver induces ROS production which results in lipid peroxidation, impaired mitochondrial function and increased DNA damage (24, 28, 60). However under physiological conditions, copper also has a role in antioxidant defense as an essential component of the Cu,Zn superoxide dismutase. We find that low dietary copper impairs the catalytic function of *Drosophila* Cu, Zn-SOD, similar to what has been observed in mammals (21), and that such flies display a dramatically shortened life span. The effect of copper concentration on life-span apparently follows a U-shape curve, as both copper starvation as well as elevated concentrations shorten life span. In both extremes, a likely cause of the premature death of adult flies is the accumulation of ROS-mediated damage. Under conditions of copper load, the shortened life span is particularly evident for metallothionein mutant males that normally express metallothionein mRNA at higher levels than females. Thus we propose that adult males depend more on the protective effects of metallothioneins than females.

We found that all four *Drosophila* metallothioneins and even a transgene with a synthetic minipromoter composed merely of four tandem copies of MREs are expressed in a very similar tissue and cell-type specific pattern in the intestine, which reproduces the known accumulation of copper or cadmium. For example, metallothioneins are expressed in the "copper cells", which are well known for their peculiar ability to accumulate high amounts of copper (18, 36), for review see (4). Cadmium was previously

shown to accumulate in the anterior midgut, in the "iron cell" region and in the posterior midgut in a pattern highly similar to the metallothionein induction we report here (33, 37). As MTF-1 is the only transcription factor known to bind directly to MREs, but ubiquitous overexpression of MTF-1 does not change the expression pattern of metallothioneins, we conclude that metallothionein expression is governed by metal distribution. This has another very practical consequence in that the metallothionein promoter driving a fluorescent protein reporter can be used as a highly sensitive and semi-quantitative biomarker for metal-content and metal transport (see also (67)). This may help to investigate the activity and function of putative metal-transporters *in vivo*.

Metallothioneins, together with glutathione, an antioxidant with relatively non-specific metal-binding ability, constitute the first line of defense against toxic effects of heavy metals in both mammals (66) and insects. As ingested metals first reach gut cells, metallothioneins in the gut probably serve to trap toxic metals and limit their distribution throughout the body. An interesting example of such trapping of toxic metal is the zinc-treatment of Wilson's disease patients who suffer from copper accumulation in the liver. Zinc treatment induces metallothionein synthesis in the intestine, and due to the metallothionein's high affinity to copper, the latter is trapped within intestinal cells and eventually excreted (58).

A remarkable finding in the present study is the autoregulation of metallothionein expression. Metallothionein promoters, used as a reporter of MTF-1 activity, are more active in metallothionein mutants. This is not due to a higher copper content, as total body copper does not differ between wild type and metallothionein mutants. Rather metallothioneins can inhibit their own expression by inhibiting MTF-1 function via the binding of free metal which otherwise would, directly or indirectly, activate MTF-1. The nature of the signal which activates MTF-1 *in vivo* is still not established. While zinc can directly bind to MTF-1, copper and cadmium are thought to activate MTF-1 indirectly and were indeed shown to do so in a cell-free model system by displacing zinc from metallothioneins, which in turn bound to MTF-1 (69). The present study however shows that metal-loaded metallothioneins are not required *in vivo* for the activation of MTF-1 by any of the heavy metals tested. It is therefore likely that zinc can be displaced from other cellular pools. Furthermore, as shown in mammals, MTF-1 regulation *in vivo* occurs at

multiple levels that also include nucleo-cytoplasmic transport, protein phosphorylation, and a conspicuous cysteine cluster towards the C-terminus of MTF-1 which might act as a metal sensor (11, 54, 55). In conclusion, the present study does not support the concept of metallothioneins playing an important role in copper accumulation and intracellular distribution, but firmly establishes their importance for coping with metal fluctuations, notably for copper homeostasis and cadmium detoxification.

Acknowledgements

We are grateful to Bruno Schmid and Antonia Manova for technical assistance, to Fritz Ochsenbein for the preparation of figures, and to Professor Jeremias Kägi, Dr. Michael Fetchko and Dr. George Hausmann for critical reading of the manuscript. D.E. is also thankful to God.

This work was supported by the Kanton Zürich and by the Swiss National Science Foundation.

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Figure Legends

Figure 1

A) Genetic map of *Drosophila* metallothioneins. Metallothionein *MtnA*, *MtnC, MtnB* and *MtnD* are in bold. The Figure is adapted from flybase (http://flybase.bio.indiana.edu/).
B) Verification of the *MtnD* knock-in allele by PCR. Note that the shorter PCR wild type product of about 0.5kb is absent in homozygous $MtnD^{dsRed}$ mutant flies.
C) Design of the *MtnD* knock-in allele. Arrows indicate the primers used for PCR.

Figure 2

Viability of *Mtn* and *MTF-1* mutants versus wild type on various metal-supplemented food. A) Absolute viability of different genotypes on normal food. Note that deviations between different strains are within the experimental error. B) The bar diagrams depict the percentage survival of mutant and wild-type (*y w*) embryos to adulthood. Survival on normal food of each genotype is set to 100%. Flies were allowed to deposit 150-300 eggs on food containing the indicated concentrations of metals, and eclosing adults were counted. Error bars represent standard deviations of several independent experiments, calculated from the number of flies in a total of 3 to 10 different tubes. C) A single metallothionein transgene can partially rescue the phenotype of a metallothionein gene family knockout with the genotype *qMtn**. Shown is the survival (in percentage of the survival of the isogenic *MtnD** genotype) of transgenic embryos to adulthood on 500 µM copper D) Sensitivity of metallothionein mutants to copper shock. Approx. 30 third instar larvae were transferred from and to the indicated type of food. Bars represent the percentage of eclosing adults. Note that there are no survivors of the $qMtn^{dsRed}$ genotype. E) Copper content of wild type and metallothionein mutant flies. Flies were grown on the indicated type of food and analyzed by ICP-MS.

Figure 3

MREs are sufficient for tissue-specific expression of metallothioneins

Flies were raised on the indicated type of food. Shown is the fluorescence of reporter transgenes with the indicated promoters driving EYFP or dsRed. Anterior is to the left. A) Expression patterns of *MtnB* and *MtnD*, two members of the MtnB subfamily, perfectly overlap. Shown is the fluorescence of the MtnB-EYFP reporter construct and the allele $MtnD^{dsRed}$ near the midgut constriction (filled arrowhead). B) Colocalization of *MtnB* promoter activity with copper cell luminescence on 1 mM copper. Shown is the expression of a MtnB-EYFP transgene. C) A synthetic promoter with MREs derived from MtnB is sufficient for tissue-specific expression and for metal induction. Note the overlap of reporter transgenes with each other and with copper cell luminescence. D) Overlap of expression near the midgut constriction of the $MtnD^{dsRed}$ knockin allele with a synthetic minipromoter composed of MREs of Ctr1B. Larvae were grown on normal food. NF = normal food.

Figure 4

Metallothioneins form a fluorescent complex with copper in copper cells of the larval midgut. Copper luminescence and DAPI staining (upper panels) and expression of $MtnD^{dsRed}$ (lower panels) in the same gut. The ubiquitous bluish fluorescence that partially masks the DAPI staining is due to autofluorescence of midgut cells (18). Note that copper cell luminescence is reduced, but not completely absent in both homozygous $qMtn^{dsRed}$ and *MTF-1* mutants. Homozygosity of the $MtnD^{dsRed}$ locus leads to stronger dsRed expression than in heterozygotes. Larvae were raised on 50 μM copper until the third instar.

Figure 5

Metallothioneins negatively regulate their own expression. A) MtnB-EYFP reporter expression in larvae heterozygous or homozygous for the *qMtn** chromosome. Anterior of third instar larvae is to the left. B) Quantification of expression levels of *MtnC* in a wild type (*y w*) and a triple Mtn (*tMtn*) mutant background, carrying the alleles $MtnA^{\Delta ATG}$, $MtnB^{\Delta ATG}$, $MtnD^{*}$. Third instar larvae were transferred for 6 hours to 500 μM copper or 4 mM zinc and transcript levels were assayed by S1 nuclease mapping. The bar diagram

represents the quantification of the gel, normalized with the signal from actin5C transcripts. C) Expression of a genomic Ctr1B-EGFP construct showing in qMtn* or heterozygous wild type larvae (qMtn*/ wt) D, E) Metallothionein transcript levels in wild type flies after a 6 hour induction period or after constant growth on the indicated type of food. D) MtnD and, E) MtnA. Quantification is relative to normal food (NF).

Figure 6

Life span of heterozygous wild type (wt/*qMtn**) and homozygous (*qMtn**) metallothionein mutant flies raised at different copper concentrations. Day 0 represents the day of eclosion. Survivorship curves indicate the proportion of a population surviving at different ages. Lifespan of heterozygous and homozygous *qMtn* mutant A) males and B) females. C) Life span of wild type flies with high copper levels. D) Lifespan of flies with copper depletion by growth on 500 μM BCS. This particular experiment was done at 26°C, all others at 28°C. E) SOD1 activity in flies raised on normal food or on low copper food. F) Metallothionein expression levels in males and females determined by S1 nuclease mapping and G) copper and zinc content of wild type flies.

MtnA

Mutant: CTCAATCAAGCGGCCGC *GGTAAGTTCGCAG*

Wild type: CTCAATCAAG**ATG**CCTTGCCCATGCGGAAGC*GGTAAGTTCGCAG*

MtnB

Mutant : TACATACAAGCGGCCGC GTGGTAC

Wild type: TACATACAAG**ATG**GTTTGCAAGGGTTGTGGAACAAGTAAGTGGTAC

MtnC

Mutant AACGAGCGGCCGC TGCAAAGGCTG

Wild type: AACGATCAAA**ATG**GTTTGCAAAGGCTG

MtnD

Mutant: CACACATTGGGTTGCAAGGCTTGCGGCCGC *GTGAGT*

Wild type: CACACA**ATG**GGTTGCAAGGCTTGTGGAACAA*GTGAGT*

Supplementary Table1: Molecular Nature of the introduced mutations. Shown is the mutant and the wild type sequence. The ATG start codon is indicated bold and underlined. Introns are italicized. Sequences that match between wild type and mutant are in red.

Supplementary Materials

Primer sequences

Cloning of gene targeting constructs

MtnA

6106: 5'-ATGGCGCGCCAAGGCATCCACAAGC

6107: 5'-ATGCGGCCGCGGTAAGTTCGCAGTCTGG

6108: 5'-ATGCGGCCGCTTGATTGAGTTGTATTTCCTCG

6109: 5'-GCACTGCTGGACGGCGGAC

MtnB

6099: 5'-ATACGCGTACAATTAAGCAAAACCGC

6100: 5'-ATGCGGCCGCGTGGTACAACGCAGCAGCAAGC

6101: 5’-ATGCGGCCGCTTGTATGTATCTATTTCTCGAC

6102: 5’-ATGGCGCGCCTGGTCTGGATTTGCGTG

MtnC

6113: 5’- ATGGCGCGCCCATGGTCAAAAGCAGG

6121: 5’- ATGCGGCCGCTGCAAAGGCTGCGGAACAAG

6122: 5’- ATGCGGCCGCTCGTTTATTGTGTTTTGATTGGTC

6123: 5’- TGACGCGTCGATGCATGTCATTTGC

MtnD

6114: 5’- ATGCGGCCGCAAGCCTTGCAACCCAaTTTGTG

6115: 5’- ATGCGGCCGCGTGAGTGTACTAGTAATCATAC

6116: 5’- CGTGCTACGTCTCATCATGTCAC

6117: 5’- ATGGCGCGCCACAGCCAAATCGATCG

Genotyping and sequencing

MtnA

6111: 5’-CATGCTGGTACATCCTGTAATCC

6182: 5’-GTGTGTAAAGCCGCGTTTCC

6183: 5’-TACATCCTGTAATCCATAAGC

MtnB

6181: 5’- GAGTTCGAGGCAATCGAAGTG

6104: 5’- GACCGCCCAGTAACAAAAAG

6184: 5’- GTAACAAAAAGTTAACGGCAG

7576: 5’- CATACAAGCGGCCGCGTGGTAC

MtnC

6124: 5’- AGCTGCCAGACTGATAATGC

6126: 5’- ACATGCGGAGCACCAAATAG

6125: 5’- TTTGCGCACGTTTTAACTTATC

6820: 5’- GTTCCGCAGCCTTTGCAGCGGC

MtnD

6118: 5’- CGCAAAGCAGTGAAAGCTC

6178: 5’- TGCAATTTCTAAAGCTAATGTGG

6177: 5’- GCTCTATAAGAATGCTGGACC

6821: 5’- GATTACTAGTACACTCACGCGGC

Supplementary Figure 1

Deletion of the ATG results in a reduction of mRNA levels. Shown is the mRNA quantification by S1 mapping. Note the faint band of full-length mRNA in *MtnA* mutant flies that is however still metal inducible.

Supplementary Figure 2.

Sensitivity of two different strains (wild type *y w* and *MtnD**) to Cu, Zn, Cd, Hg, BCS. The two strains are not isogenic and the minor differences in sensitivity between the two strains may be due to genetic background. The points depict the percentage survival of embryos to adulthood with normal food set to 100%. The experiment was performed as in Figure 2. Interestingly low concentrations of copper and cadmium or the copper chelator BCS appear to have a slight hormetic effect with higher survival than on normal food.

Supplementary Figure 3

Expression pattern of metallothionein reporter constructs. Larvae were raised on the indicated type of food. Shown is the bright field image and the corresponding fluorescence of reporter transgenes with the indicated promoters driving EYFP or dsRed. Anterior is to the left. A) MtnA-EYFP transgenic flies show expression in different parts of the larval gut, depending on the metal in the food. Intensity of expression is not comparable between different panels. B) MREs are sufficient for tissue-specificity of cadmium induction. Open arrowhead: foregut and anterior midgut, arrow: copper cell region; filled arrowhead: midgut constriction/iron cell region; dot: posterior midgut; dagger: hindgut imaginal ring; star: malpighian tubules. NF = normal food.

Figure 1

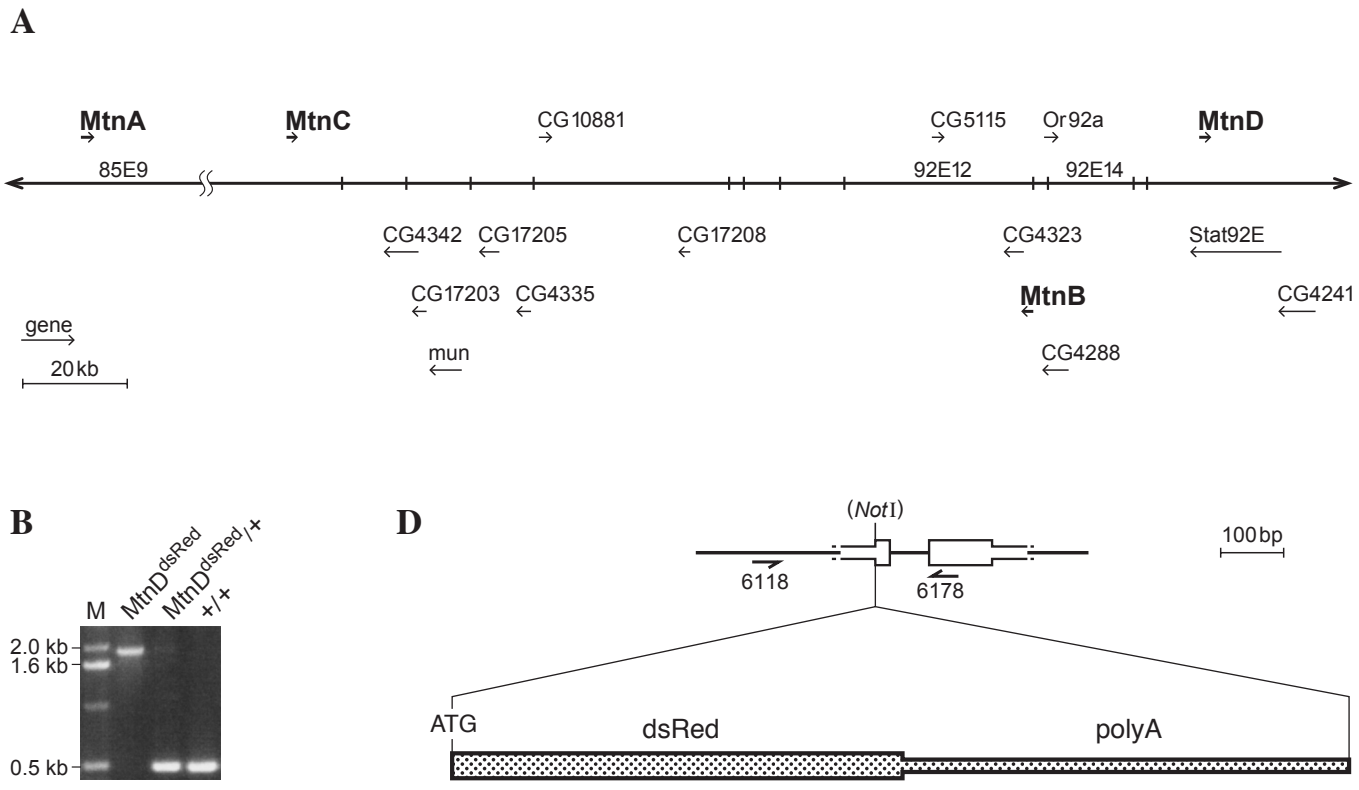

A
MtnA
MtnC
CG10881
CG5115
Or92a
MtnD
85E9
92E12
92E14
CG4342
CG17205
CG17208
CG4323
Stat92E
CG17203
CG4335
MtnB
CG4241
gene
20kb
mun
CG4288
B
M
MtnDdsRed
MtnDdsRed/+
+/+
2.0 kb
1.6 kb
0.5 kb
D
(NotI)
100bp
6118
6178
ATG
dsRed
polyA

Figure 2

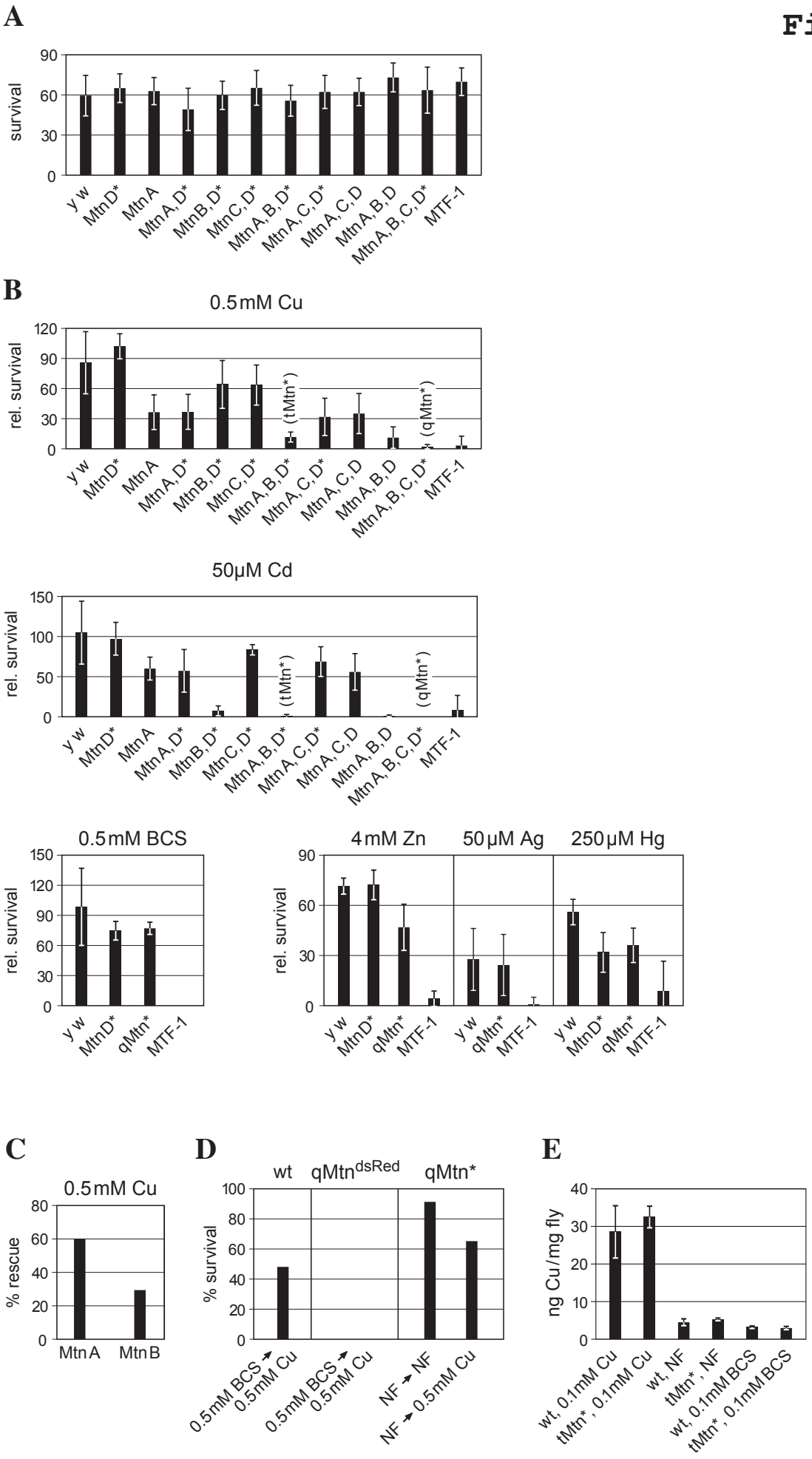
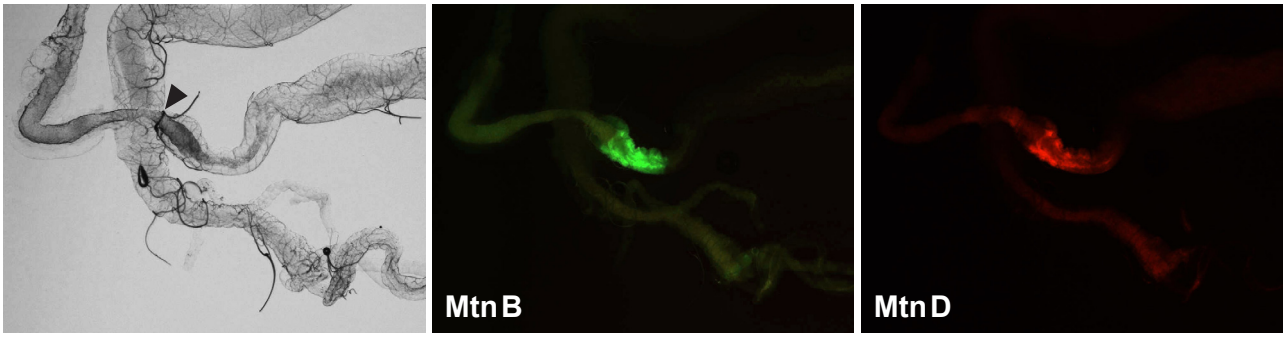

A
survival
90
60
30
0
y w
MtnD*
MtnA
MtnA,D*
MtnB,D*
MtnC,D*
MtnA,B,D*
MtnA,C,D*
MtnA,C,D
MtnA,B,D
MtnA,B,C,D*
MTF-1
B
0.5mM Cu
rel. survival
120
90
60
30
0
(tMtn*)
(qMtn*)
50µM Cd
150
100
50
0
0.5mM BCS
4mM Zn
50µM Ag
250µM Hg
qMtn*
C
0.5mM Cu
% rescue
80
60
40
20
MtnA
MtnB
D
wt
qMtn^dsRed
qMtn*
% survival
100
0.5mM BCS → 0.5mM Cu
NF → NF
NF → 0.5mM Cu
E
ng Cu/mg fly
40
30
10
wt, 0.1mM Cu
tMtn*, 0.1mM Cu
wt, NF
tMtn*, NF
wt, 0.1mM BCS
tMtn*, 0.1mM BCS

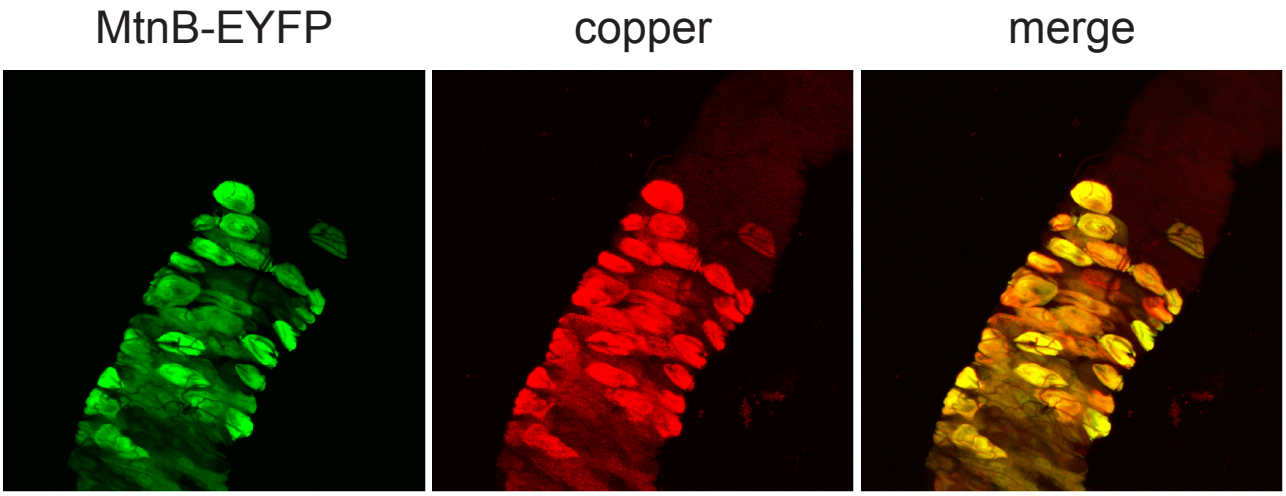
Figure 3

A

500µM Zn

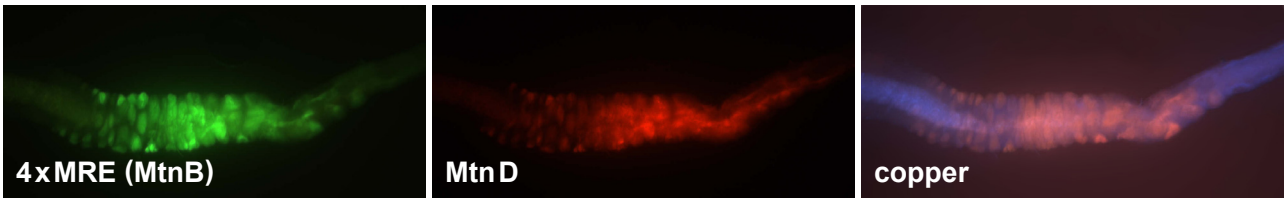


B



C

50µM Cu



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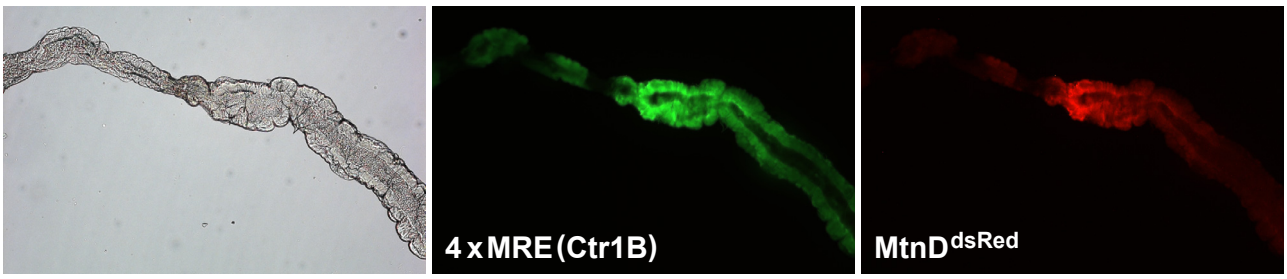


Figure 4

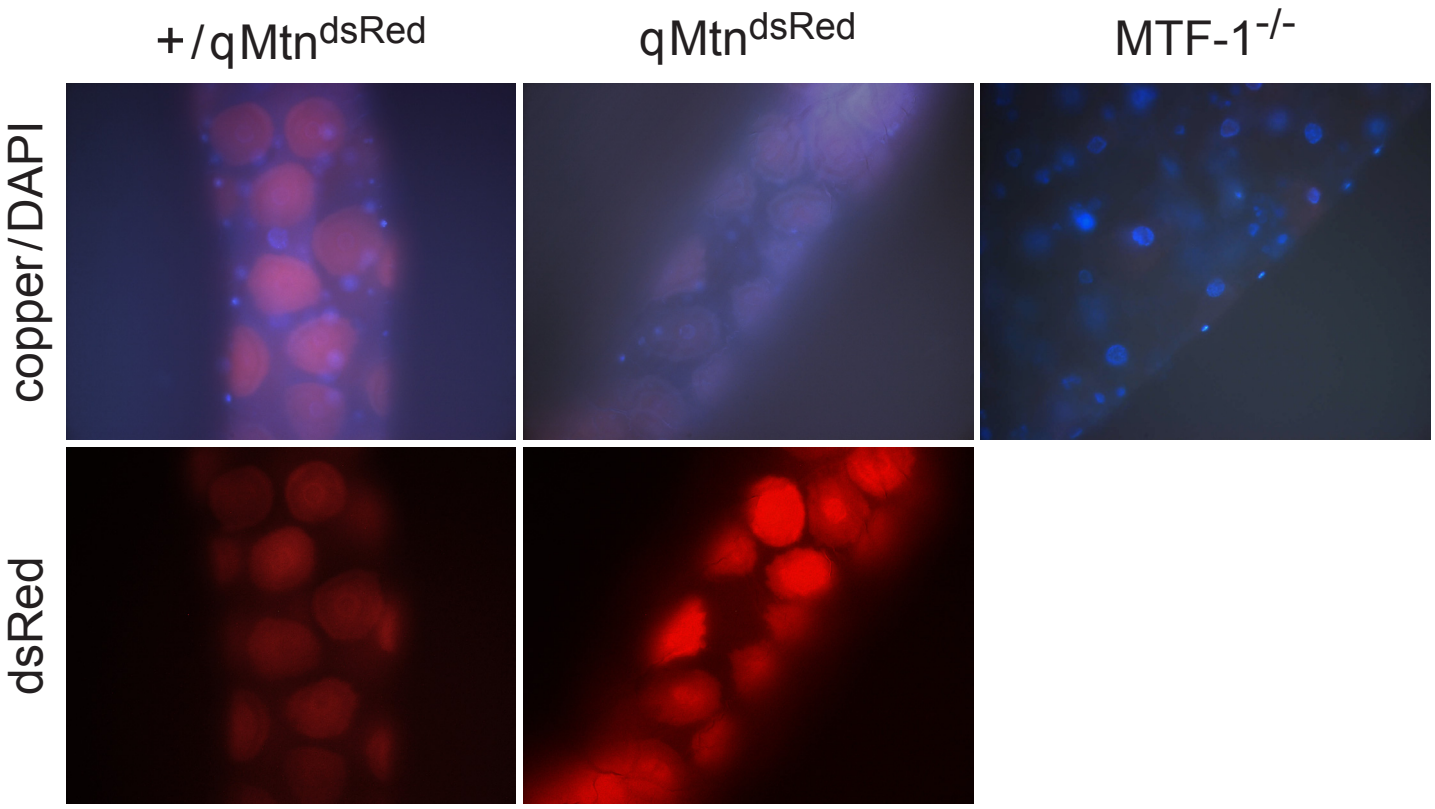


Figure 5

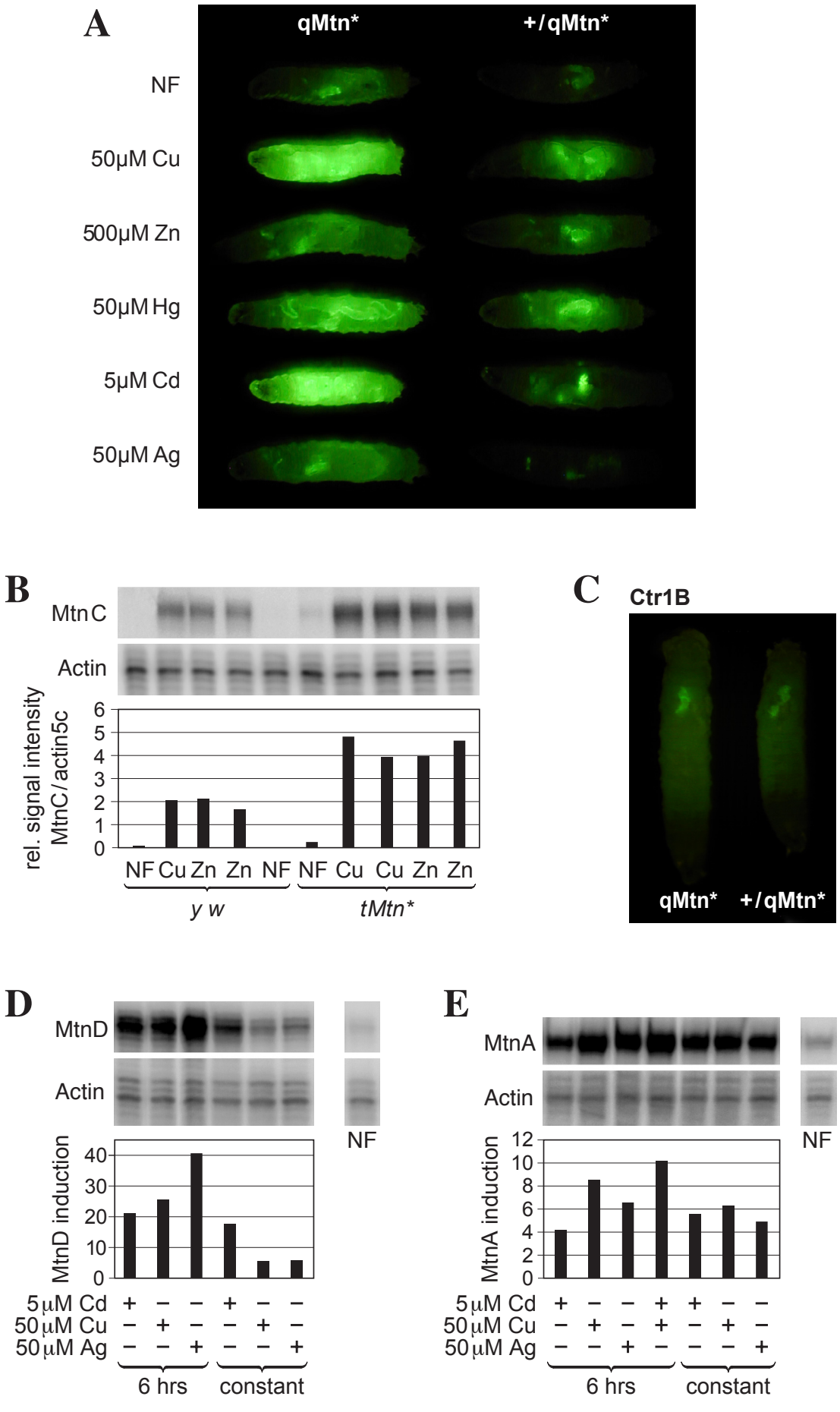
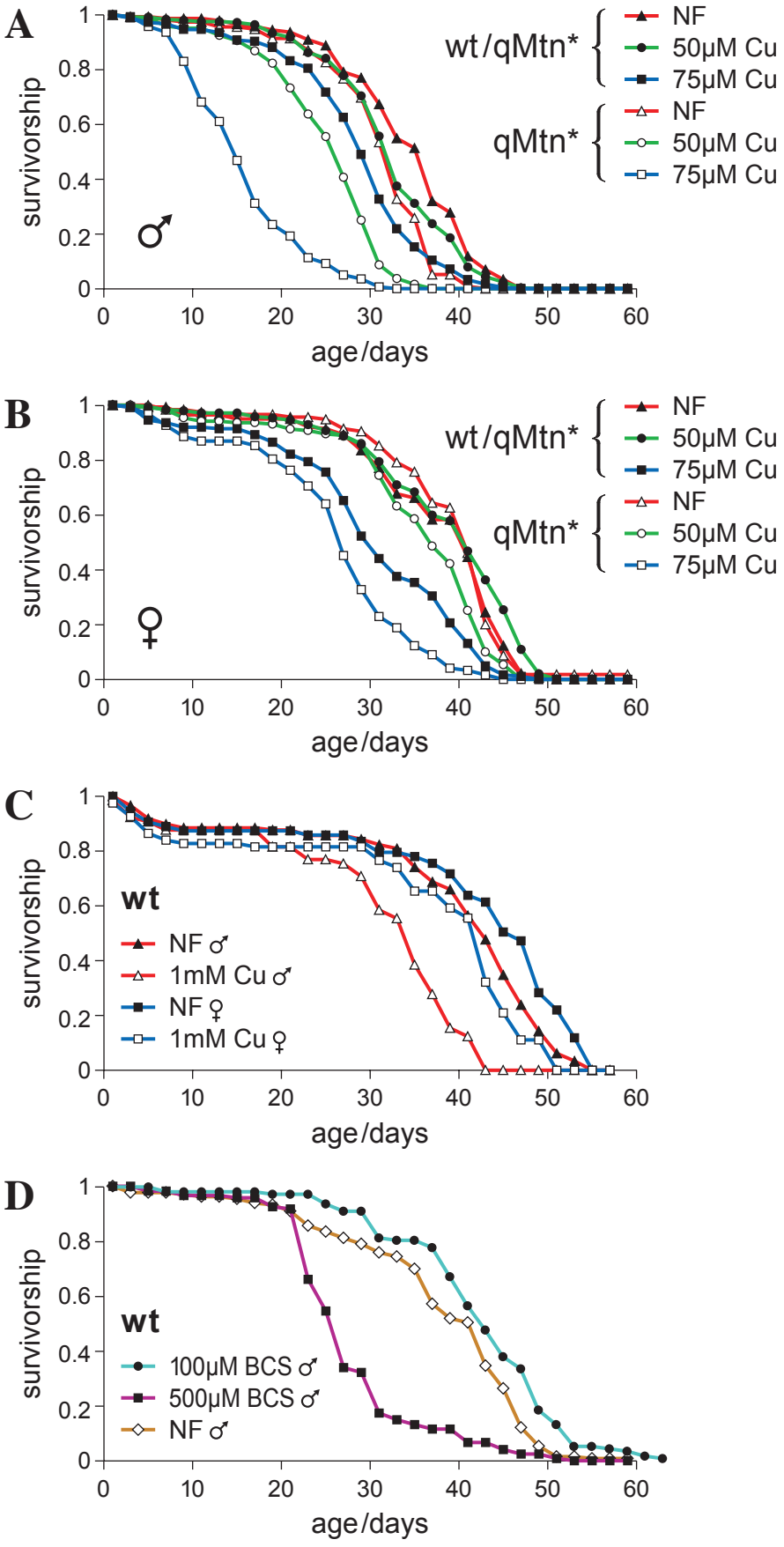

A
qMtn*
+/qMtn*
NF
50μM Cu
500μM Zn
50μM Hg
5μM Cd
50μM Ag
B
MtnC
Actin
rel. signal intensity
MtnC/actin5c
0
1
2
3
4
5
6
NF Cu Zn Zn NF NF Cu Cu Zn Zn
y w
tMtn*
C
Ctr1B
qMtn*
+/qMtn*
D
MtnD
Actin
NF
MtnD induction
0
10
20
30
40
5μM Cd + − − + − −
50μM Cu − + − − + −
50μM Ag − − + − − +
6 hrs
constant
E
MtnA
Actin
NF
MtnA induction
0
2
4
6
8
10
12
5μM Cd + − − + + − −
50μM Cu − + − + − + −
50μM Ag − − + − − − +
6 hrs
constant

Figure 6


A
survivorship
age/days
wt/qMtn*
NF
50μM Cu
75μM Cu
qMtn*
NF
50μM Cu
75μM Cu
♂
B
survivorship
age/days
wt/qMtn*
NF
50μM Cu
75μM Cu
qMtn*
NF
50μM Cu
75μM Cu
♀
C
survivorship
age/days
wt
NF ♂
1mM Cu ♂
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D
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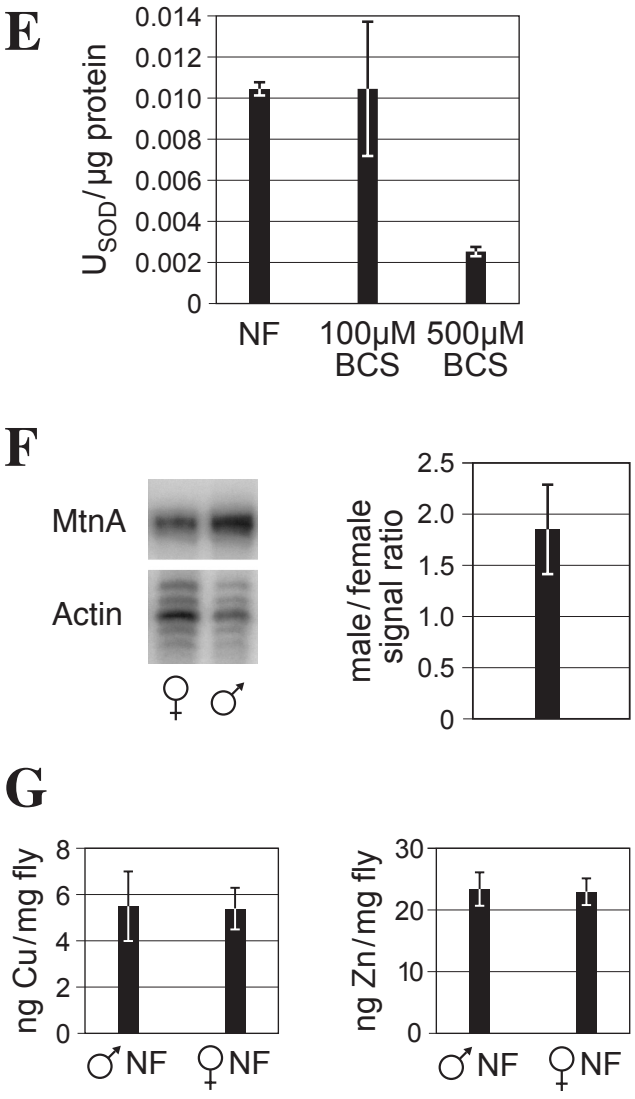
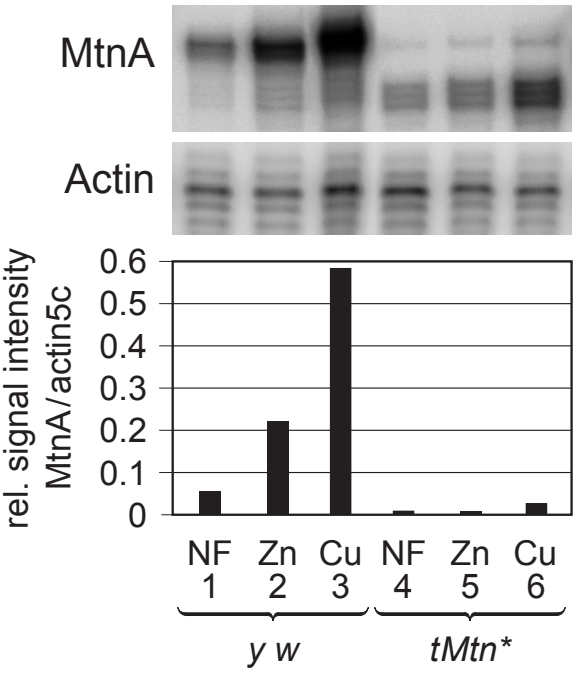
age/days
wt
100μM BCS ♂
500μM BCS ♂
NF ♂

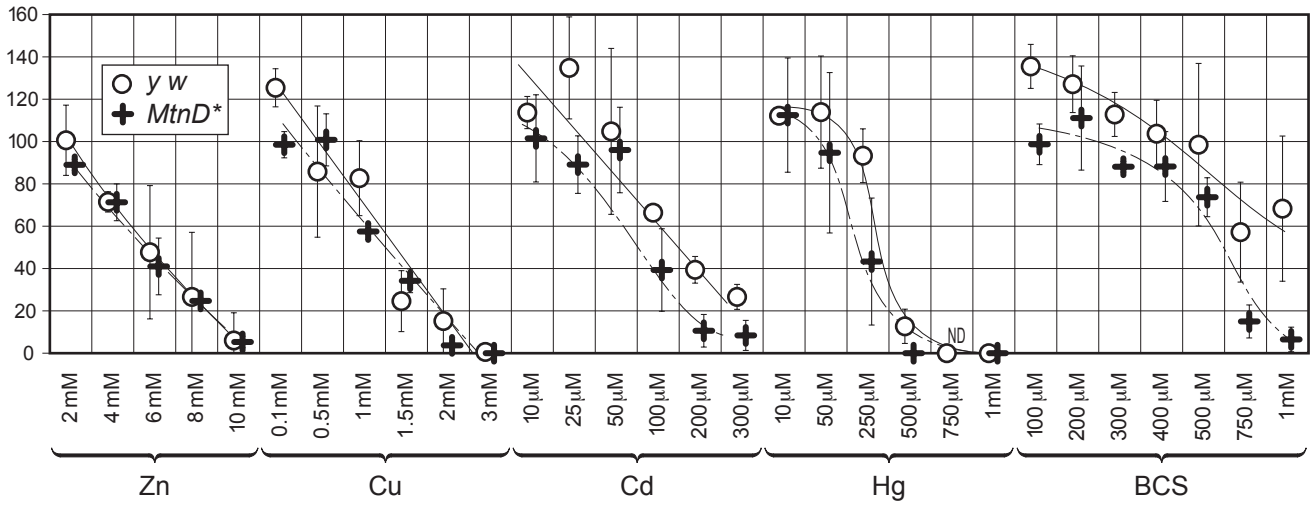
Figure 6


E
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
U_{SOD}/µg protein
NF
100µM BCS
500µM BCS
F
MtnA
Actin
♀ ♂
male/female signal ratio
2.5
2.0
1.5
1.0
0.5
0
G
ng Cu/mg fly
8
6
4
2
0
♂NF ♀NF
ng Zn/mg fly
30
20
10
0
♂NF ♀NF

Supplementary Figure 1



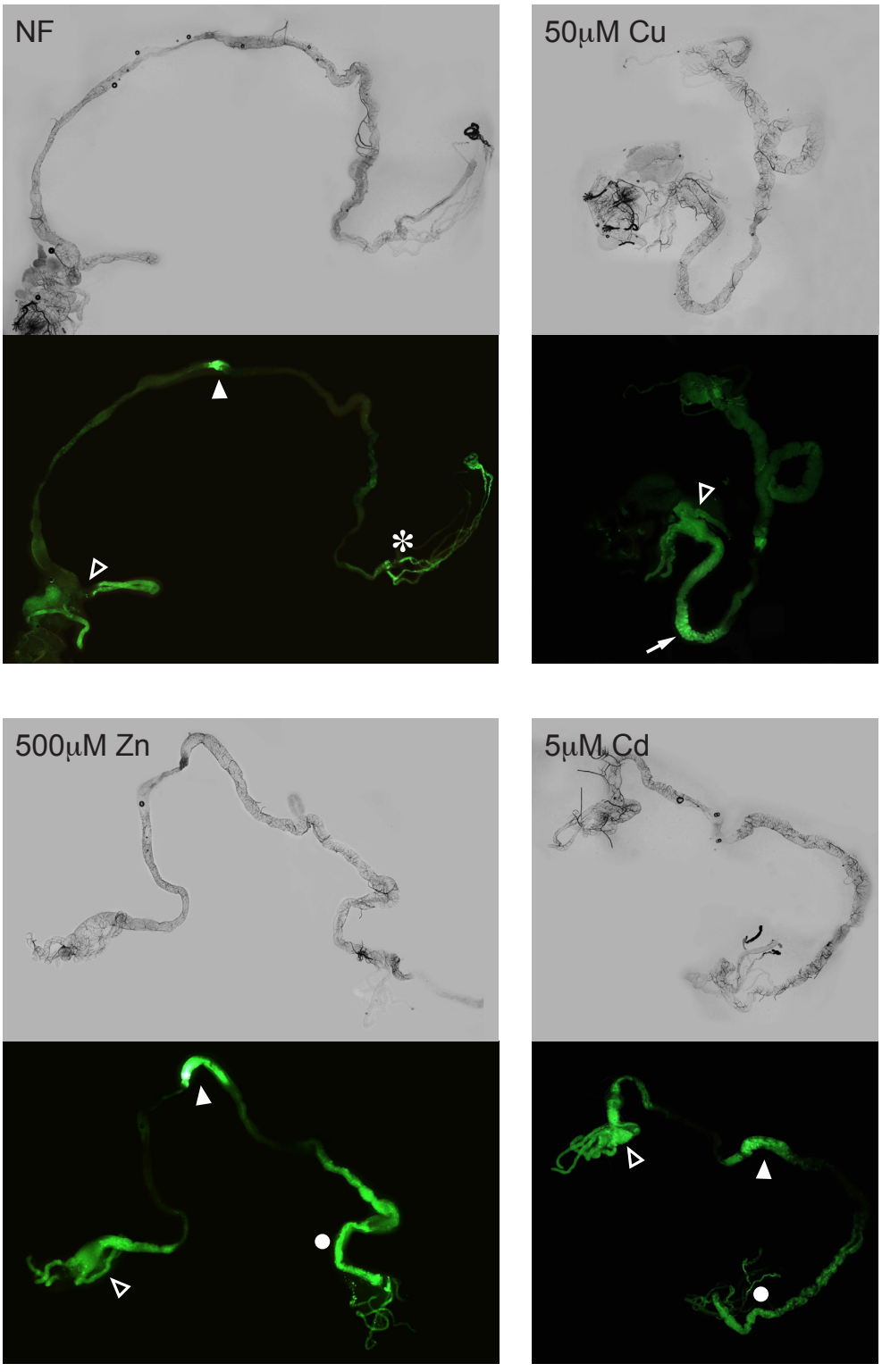
Supplementary Figure 2



Supplementary Figure 3

A

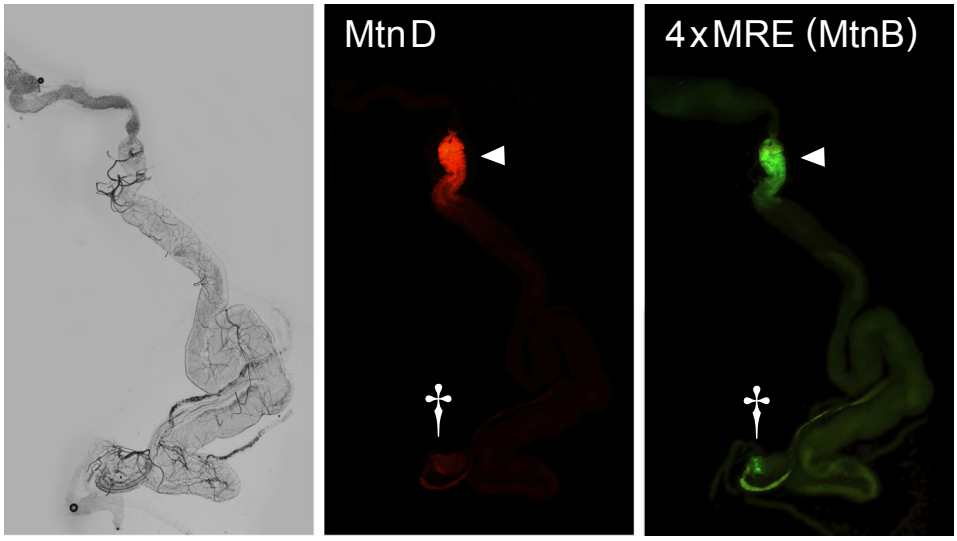
MtnA



Supplementary Figure 3

B

5µM Cd



Target genes of metal-responsive transcription factor (MTF-1) in *Drosophila*; zinc transporter *ZnT-D1* protects flies from zinc-toxicity

Hasmik Yepiskoposyan, Tim Fergestad, Dieter Egli, Oleg Georgiev, Walter Schaffner

Key words: MTF-1, MRE, heavy metals, metallothionein, ferritin, metal transport

Abstract

Metal transcription factor MTF-1 is a zinc finger protein that plays a central role in the heavy metal-induced transcriptional response from flies to humans. In mammals MTF-1 is an essential protein; mice lacking MTF-1 die *in utero* due to liver degeneration. MTF-1 knockout *Drosophila,* however is viable, yet sensitive to both low and high metal concentrations. The best characterized target genes of MTF-1 both in *Drosophila* and mammals are metallothioneins. In *Drosophila,* the copper importer *Ctr1B* is upregulated in an MTF-1-dependent manner upon copper starvation. However, neither MT-1/MT-2 double knockout in mouse nor the knockout of the *Drosophila* MT family or *Ctr1B* reproduced the whole spectrum of the MTF-1 mutant phenotype, suggesting the existence of other important target genes. Here we present results of a genome-wide search for *Drosophila* MTF-1 target genes using *in silico* and microarray-based approaches. Our search revealed several MTF-1 regulated genes, such as ferritin heavy and light chain homologs, ABC transporter *CG10505* that encodes a homolog of yeast cadmium factor and human multidrug resistance associated protein, and the zinc transporter *ZnT-D1* with a major role in zinc detoxification. This is the first study to characterize a zinc transporter in *Drosophila melanogaster*.

Introduction

Metal ions are vital for many biological processes, such as transcription, respiration and growth. However overaccumulation of essential metals such as copper or non-essential toxic metals like cadmium or lead is detrimental for the organism. Thus every organism has to accurately balance the amounts of essential metals, by providing the tissues with a greater demand for a certain metal with appropriate amounts and by fencing off the toxic or excess metal. This seemingly straightforward task is however complicated by the fact that many essential and non-essential metals share common chemical characteristics and can be taken up by the cell via the same metal transporter or bind the same substrates in the cell. Not unexpectedly, excess of an essential element can reduce the health hazard of a related toxic metal (1) on one hand, but it can also be disastrous on the other. Excess zinc, for instance, has been implicated in low copper and iron status (2). Several levels of regulation operate in the organism to maintain metal homeostasis: the gene and protein expression, transcript and protein stability, protein localization etc. The metal-responsive transcription factor-1 (MTF-1) plays a key role in the transcriptional regulation of genes involved in the heavy metal response from insects to mammals. In mammals, MTF-1 turned out to be important not only for metal homeostasis, but also for development, since mice lacking the factor die as embryos due to liver degeneration (3). In contrast, MTF-1 knockout flies ($MTF^{140\text{-}1R}$, hereafter termed MTF-1 KO) are viable under laboratory conditions but very sensitive to the elevated concentrations of heavy metals, and also to copper scarcity (4). The main target genes of MTF-1 are metallothioneins (MTs), small, cysteine-rich proteins with a high ability to bind heavy metals (4,5). In fact MTs have been discovered and shown to be important in coping with metal stress already decades ago (for review see (6)). Other target genes of MTF-1 in mammals include ZnT1, a zinc exporter, tear albumin/lipocalin, C/EBPα, placenta growth factor, selenoprotein W and N-myc downstream regulated gene 1 (7-10). However, the gene/genes responsible for the MTF-1 embryonic lethal phenotype have not been identified so far. In *Drosophila*, besides the four metallothioneins (*MtnA-D*), the copper transporter Ctr1B was shown to be MTF-1 regulated (11). Recent data show that MTs are responsible for MTF-1 sensitivity to elevated copper and cadmium concentrations, whereas Ctr1B is responsible for the low copper

sensitivity phenotype of dMTF-1 mutants. However, the genes that are important to cope with excess zinc were not identified.

For transcriptional activation MTF-1 binds through its zinc fingers to so-called metal response elements (MREs) in the promoter/enhancer regions of the target genes. MRE motifs have a core consensus TGCRCNC (where R stands for A or G and N for any of the bases) followed by less conserved GC-rich sequences. The sole presence of several MRE sequences in a synthetic promoter is sufficient for MTF-1 driven expression and metal induction (12). Also, the mutation of MRE sequences completely abolishes metal induction of MT genes (13), suggesting that MREs are both necessary and sufficient for metal-induced gene expression.

Here we present a genome-wide search for dMTF-1 target genes by a microarray transcriptome analysis and by a screen for MTF-1 binding site clusters in the *Drosophila melanogaster* genome. Several genes emerged from these studies. Ferritins and the ABC transporter CG10505 require MTF-1 only for metal induction, but not for basal level expression. The zinc exporter CG3994, which we named ZnT-D1, depends on MTF-1 also for its basal expression. Here we characterize ZnT-D1, to our knowledge the first example of zinc transporter in *Drosophila* and show that it is required for zinc tolerance in the fly.

Results

A refined MRE consensus sequence identifies novel MTF-1 target genes.
We attempted to find MTF-1 target genes by searching through the *Drosophila* genome for clusters of MREs. First, we sought for clusters of at least four MREs within a span of 300 bp using the Fly Enhancer search engine developed by Markstein et al. (14). 149 clusters fulfilled the criteria, and 105 of them were within a kilobase region upstream of the transcription (or translation) start of a gene or in the gene sequence. We found several genes harbouring clusters containing more than 5 MREs, however, some of them have no known function, and the characterized ones did not seem to be relevant in metal homeostasis or stress response. On the other hand, *MtnA*, a target gene of MTF-1 with a very high basal expression and high induction by variety of heavy metals, has only two MREs in its enhancer/promoter region. Probably not only the core consensus, but also flanking nucleotides play a role in MTF-1 mediated transcription. In order to perform a more stringent search, we had a

closer look at the MREs of metallothioneins, the best characterized target genes of MTF-1. A comparison of 18 MREs including flanking regions from four *Drosophila* metallothionein genes revealed a striking conservation of thymidine nucleotides 5′ to the consensus (Fig. 1). Also, MRE core sequences were frequently followed by a guanine and sixth nucleotide in the consensus was predominantly adenine. Next, we searched for at least two TTGCRCACG sequences in a 300 bp window. There are only twelve regions in the genome that contain three or more MRE sites conforming to our expanded consensus within a span of 300 bp or less. Three of them are associated with *MtnB, MtnC* and *MtnD*. One cluster was 668 bp upstream of the copper importer *Ctr1B*, a target gene of MTF-1 in copper scarcity (11). Another cluster containing three MREs in a 176 bp window is in the locus of the divergently transcribed ferritin genes, in fact in the first intron of the ferritin 1 heavy chain homolog (*Fer1HCH*) and 0.7 kb upstream of the ferritin 2 light chain homolog (*Fer2LCH*) transcription start (Fig.2A). Moreover, a closer look into that region revealed the presence of a fourth MRE. Another cluster of 3 MREs (within a 129 bp window) is located in the *Fer2LCH* gene, 0.5 kb upstream of the *Fer1HCH* start and a third cluster of three MREs was found further away, in the *Fer2LCH* fifth exon (Fig.2A). When we compared the *ferritin* genomic region of seven Drosophila species we observed 100% conservation of three *Fer1HCH* intronic MREs (Fig.2B). The MREs in the two exonic clusters were conserved mainly between phylogenetically closer species (*D. melanogaster, D. erecta, D. yakuba* and *D. pseubdobscura*). Next, the genome-wide search revealed a cluster of two MREs in the fifth exon of glutamate receptor gene *Glu-RI*, a gene involved in the synaptic transmission and cation transport. The other six clusters of MREs were either at least 1.5 kb away from a start of any transcription unit or in the intergenic regions of unknown genes.

A second life for ferritin genes

Ferritin is well characterized as an iron binding protein. There are also reports that it binds to a variety of heavy metals such as beryllium, copper, zinc, cadmium, lead and aluminum (15). We have recently shown that *Drosophila ferritin genes* are induced by heavy metals, with a moderate change in copper or cadmium but a considerable upregulation by zinc similar to the response to iron (16). Here we have tested the possible role of MTF-1 in the basal and metal-induced expression of *Drosophila*

ferritins. Indeed, cadmium, copper and zinc induction was abolished in the MTF-1 KO animals, whereas the iron induction remained the same (Fig.3B, C). Moreover, there is also a significant upregulation of both *ferritin* transcripts in larvae overexpressing *Drosophila* MTF-1 (OG31b) compared to the ones with only endogenous dMTF-1 (Fig.3D). These data imply *ferritins* as MTF-1 target genes in metal, notably zinc but not iron stress. The existence of several clusters of MRE motifs further suggests a direct transcriptional regulation by MTF-1.

Transcriptional profile of MTF-1 mutants

To discover new genes regulated by MTF-1 we have conducted microarray experiments with RNA from MTF-1 KO and wild type larvae kept in non-supplemented food or transferred to 50μM cadmium or 500μM copper containing food for six hours at third instar. MTF-1 target genes are expected to loose their metal regulation in the MTF-1 mutants or also to be downregulated in the mutants compared to the wt in the normal, non-supplemented food (hereafter NF).

In MTF-1 KO *Drosophila,* 42 genes were downregulated more than 2-fold (p-value cutoff 0.05) (table 1). As expected, the transcripts of metallothioneins were absent in the knockout animal and remained undetectable after metal treatment. CG4716, a gene of predicted methylene-tetrahydrofolate dehydrogenase activity, was 8.7 fold downregulated in the MTF-1 mutant. Interestingly, that gene is zinc-inducible (16) and was found to be age-regulated (17). It harbors a single MRE 63 bp upstream of the transcription start. Next, the copper importer *Ctr1B* and *CG3994*, a gene similar to mammalian zinc transporters, were found to be downregulated dramatically in the MTF-1 KO, and *CG3994* metal induction was abolished. Two triacylglycerol lipases, *CG5966* and *CG6283,* were downregulated at least four-fold in MTF-1 mutants. The latter was found to be 13 fold reduced in our previous study at the copper starvation conditions (16). The transcripts of the heat shock proteins 23 and 67Bb were also reduced in MTF-1 KO. mRNA levels of several structural proteins, transcription factors and carrier proteins were downregulated, however only two to three fold. On the other hand, only 10 genes, mostly of unknown function, were upregulated in the MTF-1 mutant (table 2). Notably, glutathione-S transferase Delta 5 (GstD5) was five fold induced. Gsts are stress inducible enzymes involved in the glutathione-mediated

detoxification pathway. Probably this member of Gst Delta family was induced to compensate for the loss of MTF-1.

Next, we checked the expression of the metal inducible genes in the MTF-1 mutants. Several genes encoding glutathione-S transferases, cytochrome P450 proteins, as well as few transporter proteins such as ATP-binding cassette (ABC) transporter CG10505 are induced by heavy metals (16). Transcripts of all these genes were not significantly changed in the MTF-1 mutant larvae compared to the wt when fed with normal food. However, after the transfer into the metal containing food we did not observe an induction of *CG10505* in the KO. This behavior resembles that of *ferritin* genes suggesting a role of MTF-1 only in elevated concentrations of heavy metals.

CG10505, a homolog of yeast cadmium factor, contributes to metal homeostasis

CG10505 is a member of ABC-transporter family. Several members of that family in other organisms as yeast cadmium factor (YCF) in *S. cerevisiae* or multidrug resistance associated protein (MRP) in human confer resistance to cadmium or to various cytotoxic drugs (ref.). Screening the promoter/enhancer region of the gene revealed the presence of one MRE 0.8 kb upstream of the annotated translation start.

We tested the expression of *CG10505* by the S1 nuclease protection assay. In three assays we repeatedly saw a loss of metal induction in the MTF-1 mutant. A representative gel and the quantification of bands are shown in Fig.4A. Zinc turned to be the best inducer of the gene at the concentrations of metals used. To test the function of the gene in vivo we made Gal4 inducible overexpression constructs by fusing the cDNA of *CG10505* to a promoter that contains four tandem copies of Gal4 upstream activating sequences (UAS) from yeast. The cross of transgenic flies with a fly strain that carries actin-Gal4 did not yield any obvious phenotype in normal food or when challenged with different concentrations of zinc, cadmium or copper. We then tested if the overexpression of this gene could rescue the metal sensitivity phenotype of MTF-1 mutants. MTF-1 mutants do not survive 5mM zinc, 100uM cadmium or 500uM copper (4). However, if animals KO and wt for MTF-1 are raised in the same tube, the KO genotypes suffer even more probably due to low competitiveness. To test for rescue of the MTF-1 KO by CG10505 we brought UAS-CG10505 and actin-Gal4 driver transgenes into the MTF-1 mutant background and let

the offspring of crosses grow up in different concentrations of metals. Thereby we could observe a partial rescue of zinc sensitivity phenotype; for example, a few MTF-1 homozygous KO flies eclosed from 2mM zinc food only if they overexpressed CG10505 (Fig.4B). CG10505 expressing flies had a clear advantage at lower zinc concentrations. However, the KO did not benefit of this overexpression in cadmium or copper containing food. Based on the transcript quantification and genetic rescue results, we conclude that *CG10505* is a target gene of dMTF-1 with a potential role in zinc homeostasis.

***ZnT-D1* zinc transporter is required for zinc tolerance**

Next, we tested *CG3994,* a gene that was more than six-fold downregulated in the MTF-1 KO larvae compared to wt. Indeed, also in the S1 nuclease protection assay the transcripts of *CG3994* were virtually absent in the MTF-1 KO larvae compared to the wt kept in NF or food supplemented with cadmium, copper or zinc (Fig.5A). Based on the sequence similarity to the characterized mammalian zinc transporters, *CG3994* had previously been annotated as a zinc-transporter like gene in the FlyBase. This sequence similarity, metal inducibility and the characteristics described below prompted us to name it ZnT-D1, including it into the family of zinc exporter proteins. In *D. melanogaster*, two isoforms of the gene, *ZnT-D1A* and *ZnT-D1B*, are annotated. They differ in the first few exons and share the fifth to tenth exons (Fig.5B). The TMpred software developed for a prediction of membrane spanning regions of the proteins suggested 6 strong transmembrane helices in both isoforms of the ZnT-D1 protein. A search for the possible MREs revealed two of them in the promoter/enhancer region of each isoform.

The *ZnT-D1A* isoform turned to be the major one expressed in all developmental stages, predominantly in adult females (Fig.5C). Neither isoform could be detected in cultured S2 cells. In larvae, the expression of the gene was very low; in this stage of rapid growth zinc is probably of great need, and the lowering of the zinc exporter expression would help to maintain zinc sources. Next, we checked the metal induction of each isoform in the S1 nuclease protection assay. Here also, the *ZnT-D1A* was strongly induced upon cadmium and zinc challenge, whereas we could hardly detect any mRNA from ZnT-D1B (Fig.5D). To test the functions of the gene in vivo, we made Gal4 inducible overexpression constructs by fusing the cDNAs of both

isoforms to the UAS promoter. Transgenic flies were viable after crossing to actin-Gal4 driver. We were curious to see if ZnT-D1 overexpression would give any advantage to the flies in elevated concentrations of metals. For that, we separately crossed several transgenic lines of UAS-ZnT-D1A and UAS-ZnT-D1B to the actin-Gal4 driver flies in the food supplemented with different concentrations of heavy metals. Flies overexpressing either isoform of ZnT-D1 were particularly resistant to high zinc (Fig.6). Flies benefit the overexpression of the Znt-D1A isoform also in the cadmium food (Fig.6A). Unexpectedly, flies overexpressing the ZnT-D1B isoform were extremely sensitive when challenged with cadmium or copper compared to wt (Fig.6B). One possibility might be that high ectopic expression of ZnT-D1B depletes the organism from zinc. In the normal food this would have no effect, whereas an addition of cadmium or copper can be fatal for the cell/organism. Indeed, the addition of moderate zinc (1mM) to the cadmium or copper food could partially rescue the fraction of ZnT-D1B overexpressing flies (data not shown).

We used similar transgenic flies with a ZnT-D1-GFP fusion under the UAS promoter. When crossed to the actin-Gal4 driver, the larval gut showed specific fluorescence in the proventriculus, gastric caeca and hindgut (Fig.7A). The plasma membrane showed a green fluorescnence in agreement with the ZnT-D1 role in transport (Fig.7B,C). The results were similar for both isoforms. However, the specific expression pattern driven by ubiquitous actin-Gal4 suggests a post-transcriptional regulation of *ZnT-D1*. The addition of zinc did not affect the intensity or localization of the protein (not shown).

Next, we generated a knockout of *ZnT-D1* gene by the homologous recombination technique (4,18). The knockout of *ZnT-D1* is viable with no apparent phenotype at normal laboratory conditions. Preliminary results show that the mutant is very sensitive to high zinc concentrations; adult flies die in 5mM zinc food within 2 days. Also, the eggs do not develop in 5mM zinc, except for a few survivors, that, however, die at first instar stage. *ZnT-D1* mutants appear not more sensitive to copper or cadmium load than wildtype (data not shown).

Materials and methods

Fly food and RNA extraction

Animals were raised on standard cornmeal molasses-based food. In the 3rd larval instar (fourth day of the development) the animals were transferred from normal to supplemented food containing 0.05 mM $CdCl_2$, or 5 mM $ZnCl_2$. For the microarray and S1 nuclease protection assays RNA was extracted after six hours of feeding on the supplemented food. For the experiment shown in Figure 5C, RNA has been extracted from the animals in the different development stages or from S2 cells (see figure legend). To control for the handling of the larvae during transfer to supplemented food, the normal food controls were also removed at 3rd instar and transferred for the last six hours to normal food. Total RNA was extracted using TRIzol reagent (Life Technologies).

Fly stocks and genetics

The following transgenic and mutant fly strains have been used in this study:

MTF-1$^{140\text{-}1R}$/ MTF-1$^{140\text{-}1R}$

UAS-ZnT-D1A (several lines),

UAS-ZnT-D1B (several lines),

UAS-ZnT-D1B/CyO, MTF-1$^{140\text{-}1R}$/ MTF-1$^{140\text{-}1R}$

UAS-ZnT-D1A-GFP,

UAS-ZnT-D1B-GFP,

UAS-CG10505 (several lines),

UAS-CG10505/CyO, MTF-1$^{140\text{-}1R}$/ MTF-1$^{140\text{-}1R}$

Actin-Gal4/TM3ser;y^{+},

Actin-Gal4; MTF-1$^{140\text{-}1R}$/TM6By^{+}.

MTF-1$^{140\text{-}1R}$ is designated as KO throughout the manuscript.

The ZnT-D1 gene targeting has been done as described earlier with minor modifications (4).

GFP fusion protein expression analysis and microscopy

For ZnT-D1-GPF (ZnT fused to GFP) fusion protein analysis, flies were allowed to deposit eggs in the food and raised until third instar larvae. Larvae were dissected and

analyzed under a Leica DRB fluorescence stereomicroscope (Fig.7A) or a Leica TCS SP spectral confocal microscope (Fig.7 B&C).

S1 nuclease protection assay

Nuclease S1 protection assay with was performed as described (19). The dried gels were exposed to storage phosphor screens and analyzed using a PhosphorImager (Molecular Dynamics).

Microarray

Microarray experiments were done in triplicates using a pool of RNA from at least 30 animals for each assay. cDNA was synthesized from larval total RNA with SuperScript reverse transcriptase (Invitrogen/Life Techn. cDNA Synthesis Kit) using T7-(T)24 primer 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTT-TTTTTTTTTTTT-3'. The resulting cDNA was purified with Phase Lock Gels and concentrated by ethanol precipitation. Synthetic double-stranded cDNA was in vitro transcribed into biotin-labeled cRNA with biotinylated 11-CTP and 16-UTP (Ambion MEGAscript T7 kit). Biotin-labeled cRNA was then isolated with an RNeasy Mini Kit (Qiagen). 15 μg adjusted cRNA was taken for fragmentation. 11.5 μg fragmented cRNA (50-200-nucleotide fragments) was used to probe the *Drosophila* Genome Array (Affymetrix), which contains more than 13,500 mRNA transcripts from known *Drosophila* genes and predicted ORFs. Probe arrays were treated with streptavidin, anti-streptavidin goat antibody, biotinylated goat IgG antibody, and stained with streptavidin phycoerythrin. Arrays were scanned twice with an Agilent G2500 Genome Array Scanner.

Software and Statistical Analysis

Raw data processing was performed using the Affymetrix Microarray Suite Ver. 5.0 (MAS5) Software. After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS5 algorithm (20). In order to compare the expression values of the genes from chip to chip, global scaling was performed resulting in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500 as described in the Statistical Algorithms Description Document (Affymetrix, 2002). Quality control

measures were considered before performing the statistical analysis. These included adequate scaling factors (between 10 and 17 for all samples) and appropriate total number of "Present Calls" per chip (26-30%) calculated by application of a signed-rank call algorithm (21). Furthermore, the optimal 3′/5′ hybridization ratios (around 1) for the housekeeping genes (GAPDH, actin) as well as for the spike controls (BIOB, BIOC, CREX, BIODN), added as hybridization controls into the hybridization cocktail were taken into consideration. After filtering of genes with unreliable expression, using the Cross-Gene Error Model implemented in the Gene Spring software 5.1. (Silicongenetics, 2003) unequal variance t-test was applied to detect significantly differentially expressed genes. In general, a significance level of 0.05 was chosen. Furthermore, the signal-rank call algorithm from the MAS5 software (21) was applied as an additional filter. Within one comparison of two conditions, each gene was taken into account for further analysis if the algorithm attributed "Present Calls" to at least 50% of the values.

Discussion

The knockout of *Drosophila MTF-1* gene results in an altered expression of more than 50 genes in the microarray experiment. This, however, does not result in an obvious phenotype unless the animal is challenged with non-physiological amounts of heavy metals; copper, zinc, cadmium or mercury load on one hand and copper scarcity on the other (4,22). Recently we have shown that the low copper sensitivity of *MTF-1* KO is due to a loss of regulation of the copper importer *Ctr1B* (11). Also, recently in our laboratory a fly lacking all four metalothioneins (qMtn) has been generated. These flies have similar sensitivity to cadmium or copper excess as MTF-1 KO flies do. Upon zinc load, however, qMtn hardly shows any phenotype different from wild type flies. Here we describe novel target genes of MTF-1 and propose them to be responsible for *Drosophila* zinc resistance. Most importantly, the knockout of *ZnT-D1* by homologous recombination displays a similar sensitivity to zinc as MTF-1 KO flies. In addition, *ferritin* and *CG10505* genes also contribute to metal homeostasis, in that both are induced by metals in an MTF-1-dependent manner and the latter also partially rescues MTF-1 mutant's zinc sensitivity, when overexpressed. The overexpression of ZnT-D1 gives a clear advantage under conditions of zinc excess. However, it did not rescue the zinc sensitivity of the MTF-1 mutants. This can be explained in several ways: firstly, there are several zinc-protective MTF-1 dependent genes (*CG10505*, and probably *ferritins*), and the contribution of each may be important for measurable protection against zinc load. Second, our overexpression system drives the *ZnT-D1* promoter in the whole organism, whereas a tissue- and time-specific expression of it might be important for proper protection.

It is interesting to note that mouse ZnT1 has also been found to be induced both by cadmium and zinc treatment, and that this regulation is MTF-1 dependent (7). Thus not only the metallothionein regulation but also that of zinc transporters is conserved between flies and mammals. Possibly several other common target genes await characterization. Noteworthy, two triacylglycerol lipases (CG6283 and CG5966) are downregulated in the MTF-1 KO larvae more than four-fold. Triacylglycerol lipases catalyze the hydrolysis of triacylglycerols; the latter comprise almost 90% of dietary lipids and are the major storage form of metabolic energy in humans. The malfunction of these enzymes has been linked to various hepatic and pancreatic diseases. It is tempting to speculate that similar gene(s) might be controlled

by MTF-1 in mammals, and the misregulation of that gene(s) could be responsible for the lethal hepatic degeneration phenotype in MTF-1 knockout mouse embryos.

Acknowledgements

We thank the Functional Genomics Center Zurich (FGCZ) for advice and for financial support. We are grateful to Dr. Ulrich Wagner for assistance in microarray data analysis, and to Andrea Patrignani and Bruno Schmid for technical support. This work was supported by Kanton Zürich and by the Swiss National Science Foundation.

Figure 1

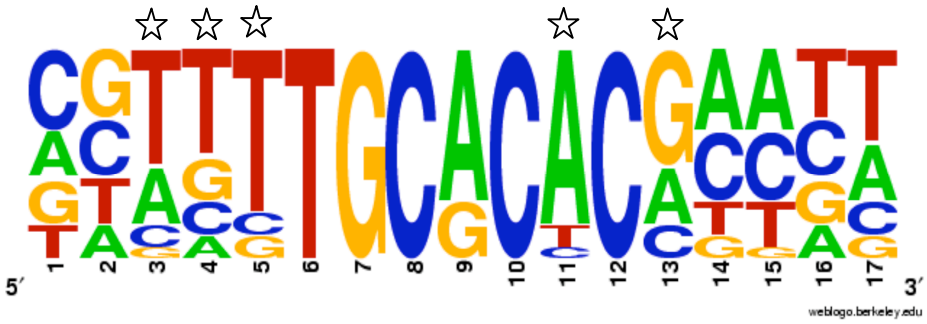
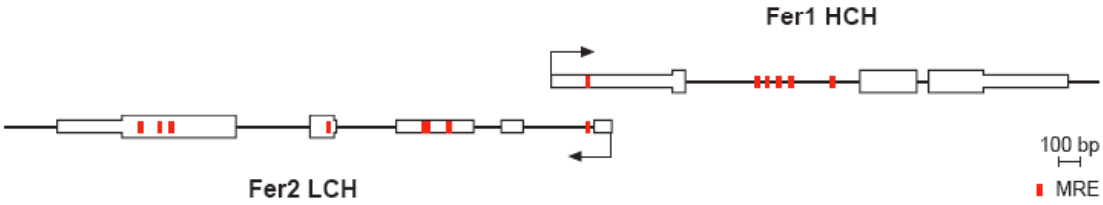


Fig. 1 Expanded MRE consensus derived from 18 MREs associated with *Drosophila* metallothionein genes. The core MRE is TGCA/GCNC, five flanking nucleotides 5′ and 3′ to the core consensus are shown. The stars indicate the nucleotides that occur in the position at least in 9 out of 18 compared sequences (at least 50% occurrence).

Figure 2

A.



B.

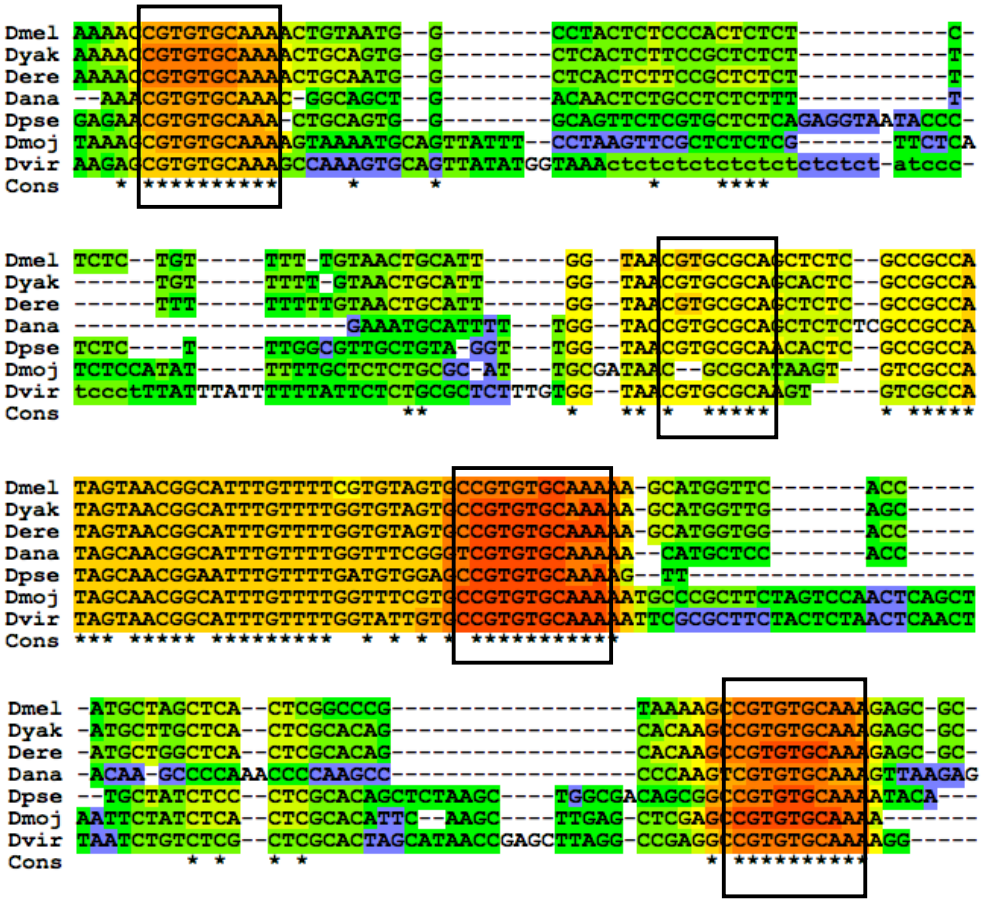


Fig. 2 The organization of *Drosophila* ferritin genes and MREs in the ferritin region. A. The genomic region of *ferritin* genes in *D. melanogaster*. The arrows indicate the transcription starts. Exons are shown as rectangles, introns as lines. The narrow rectangles correspond to the UTRs, the large ones to translated regions. **B.** The comparison of *Fer1HCH* intronic MREs across 7 *Drosophila* species: *D. melanogaster, D. yakuba, D. erecta, D. ananassae, D. mojavensis* and *D. virilis*. The black rectangles highlight MREs, the stars indicate 100% conservation.

Figure 3

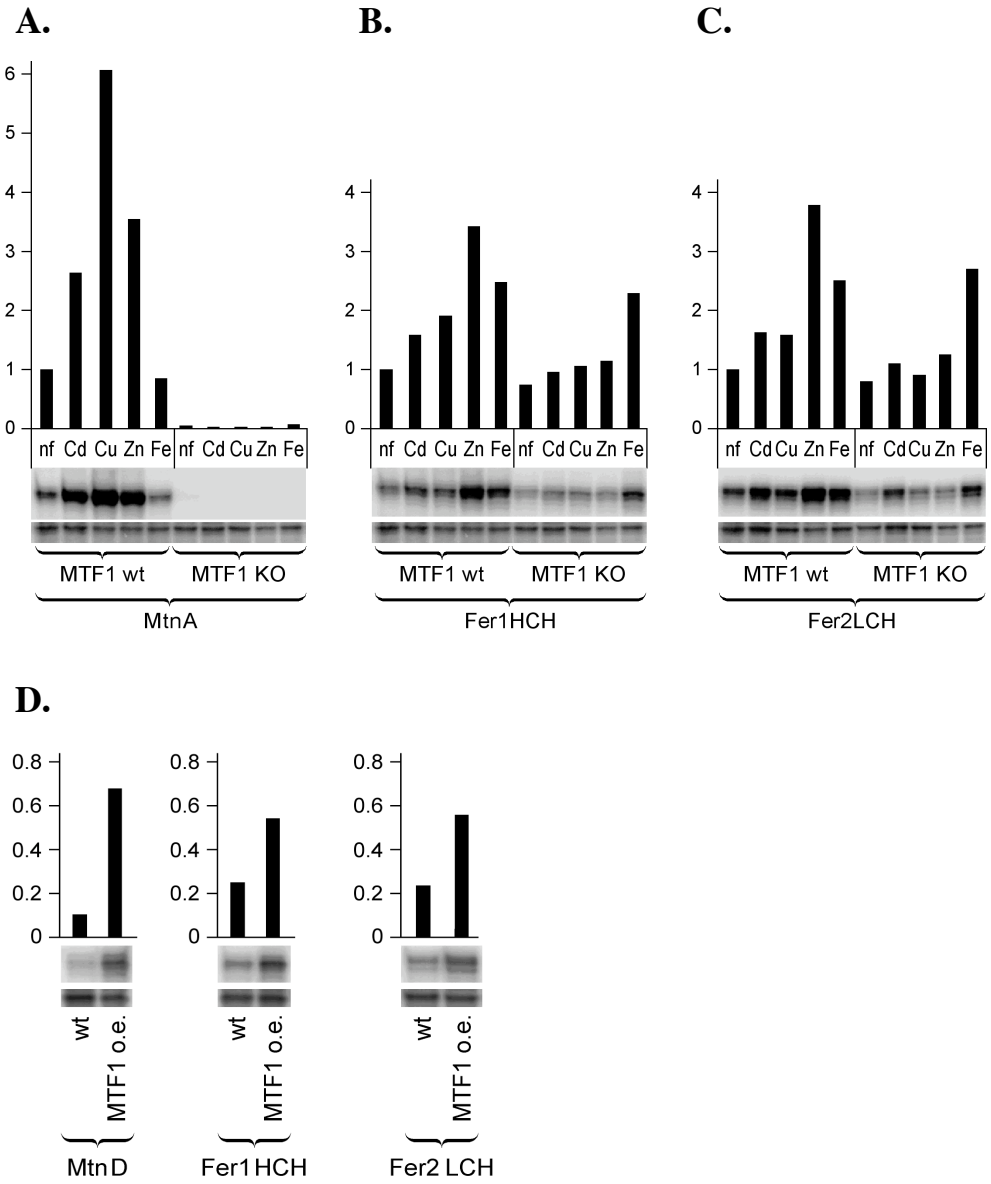
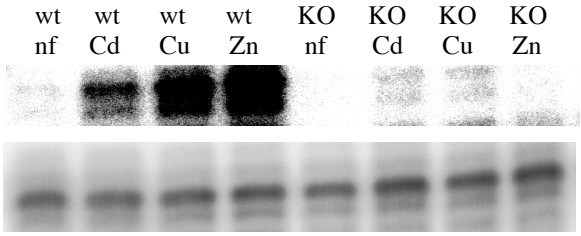


Fig. 3 Transcript quantification by S1 nuclease protection assay. Transcripts of *MtnA* (**A**), *Fer1HCH* (**B**) and *Fer2LCH* (**C**) were determined at different food conditions. nf = normal food, Cd = 50 uM $CdCl_2$, Cu = 500 uM $CuSO_4$, Zn = 5 mM $ZnCl_2$, MTF1 wt = RNA from larvae wild type for MTF1, MTF1 KO = RNA from MTF1 knockout larvae. **D.** Both *ferritin* genes as well as *MtnD* are induced in *Drosophila* that overexpress MTF1 under the *tubulin* promoter. MTF1 o.e. = overexpression of the MTF1. Reference is actin5.

Figure 4

A.



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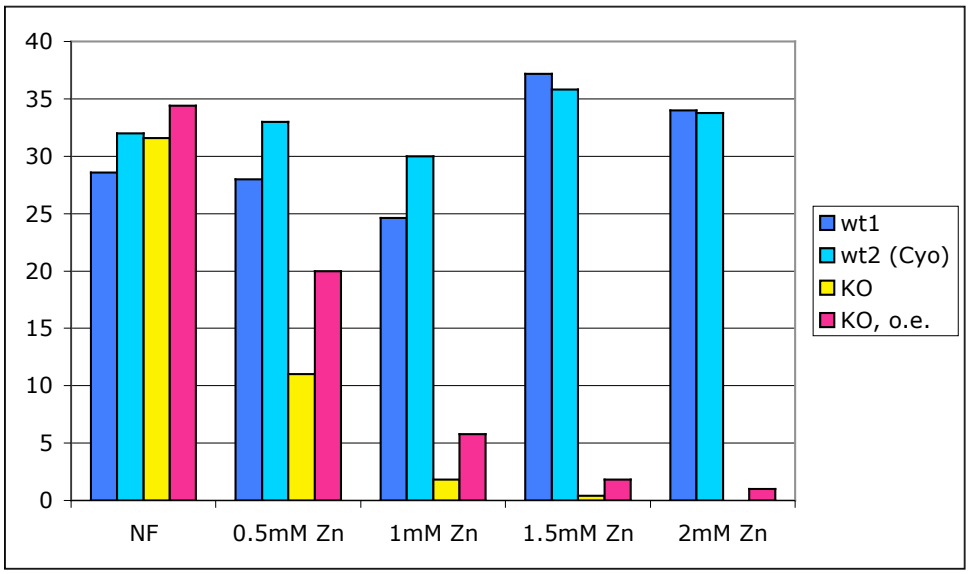


Fig. 4 ABC transporter CG10505: transcript quantification and overexpression in the flies A. S1 nuclease protection assay for transcripts of the ABC transporter *CG10505*. nf = normal food, Cd = 50 uM $CdCl_2$, Cu = 500 uM $CuSO_4$, Zn = 5 mM $ZnCl_2$, MTF1 wt = RNA from larvae wild type for MTF1, MTF1 KO = RNA from MTF1 knockout larvae. Reference: tubulin85. **B.** Partial rescue of MTF-1 mutant flies zinc sensitivity phenotype by CG10505 expression. The average number of flies from five independent experiments is shown. KO = MTF-1 knockout flies, o.e. = overexpression of CG10505 with actinGal4 – UAS-CG10505 system

Figure 5

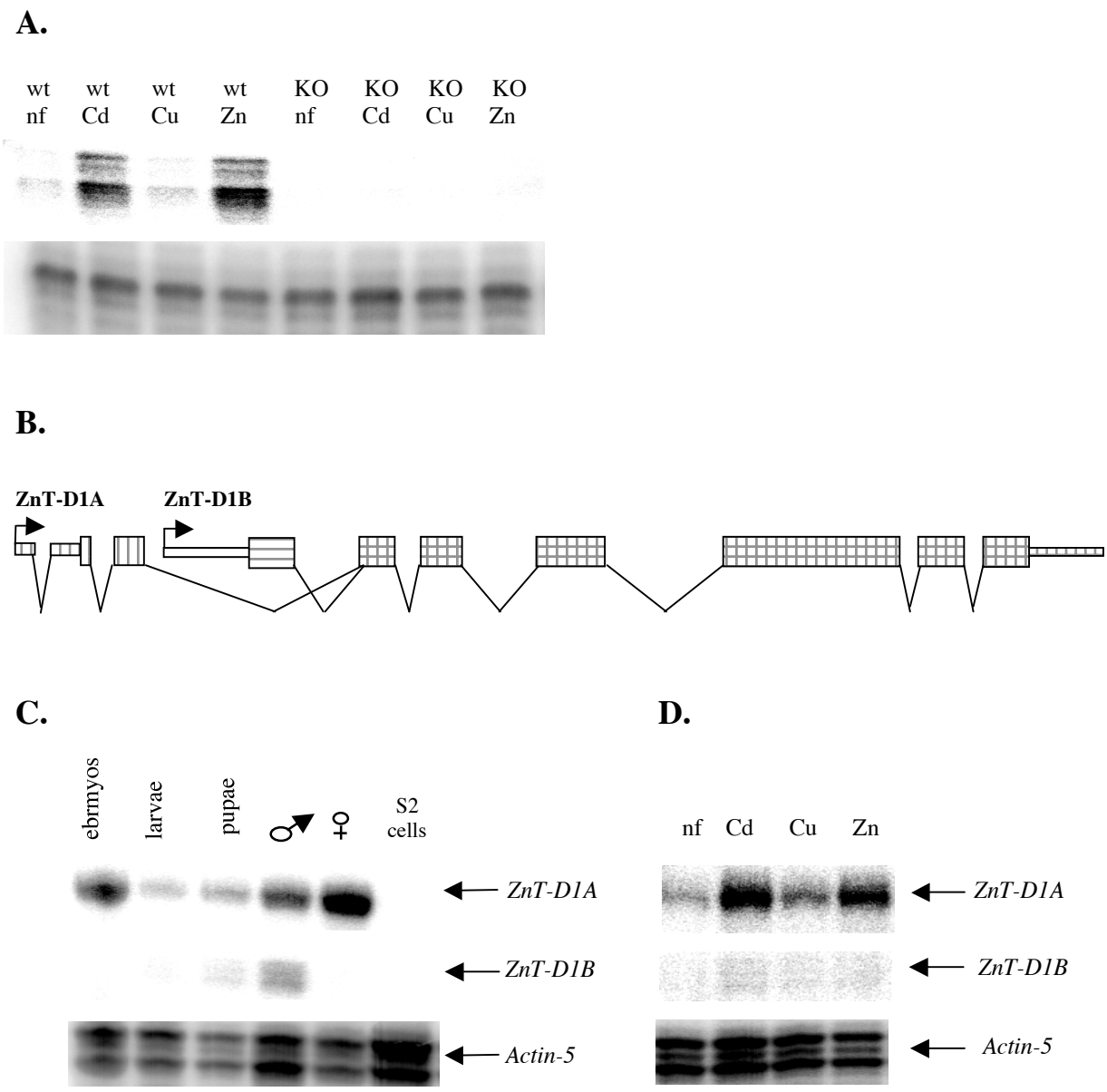
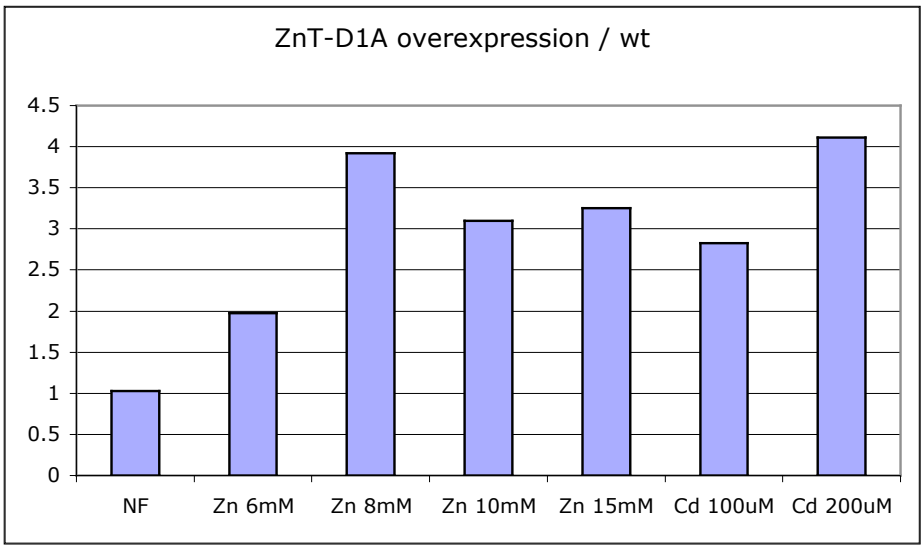


Fig. 5 Zinc transporter ZnT-D1: expression and genomic organization of two isoforms. **A**. S1 nuclease protection assay for the zinc transporter *Znt-D1*. An S1 oligonucleotide was used that is complementary to the common region of both *ZnT-D1A* and *ZnT-D1B* isoforms. nf = normal food, Cd = 50 uM $CdCl_2$, Cu = 500 uM $CuSO_4$, Zn = 5 mM $ZnCl_2$, MTF1 wt = RNA from larvae wild type for MTF1, MTF1 KO = RNA from MTF1 knockout larvae. Reference: Tubulin85. **B.** *ZnT-D1* gene structure. The arrows show the transcription starts. The narrow rectangles correspond the untranslated regions, the large ones show translated regions, the rectangles filled with double grid are the shared exons for both isoforms. **C.** S1 nuclease protection assay for the *ZnT-D1A* (upper panel) and *ZnT-D1B* (lower panel) isoforms with RNA from different developmental stages and S2 Schneider cells. **D.** The response of the ZnT isoforms to cadmium, copper and zinc.

Figure 6

A.



B.

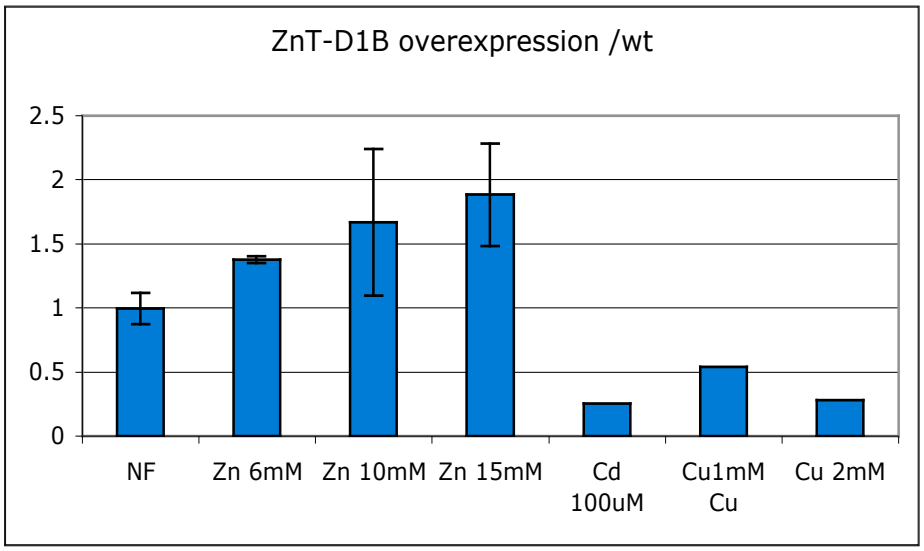


Fig. 6 Overexpression of the zinc transporter ZnT-D1 with actin-Gal4 system. Flies overexpressing the zinc transporter ZnT-D1A (**A**) and ZnT-D1B (**B**) are more resistant to zinc load than wildtype. The bars represent the ratio of the zinc-transporter overexpressing flies over the wildtype flies.

Figure 7

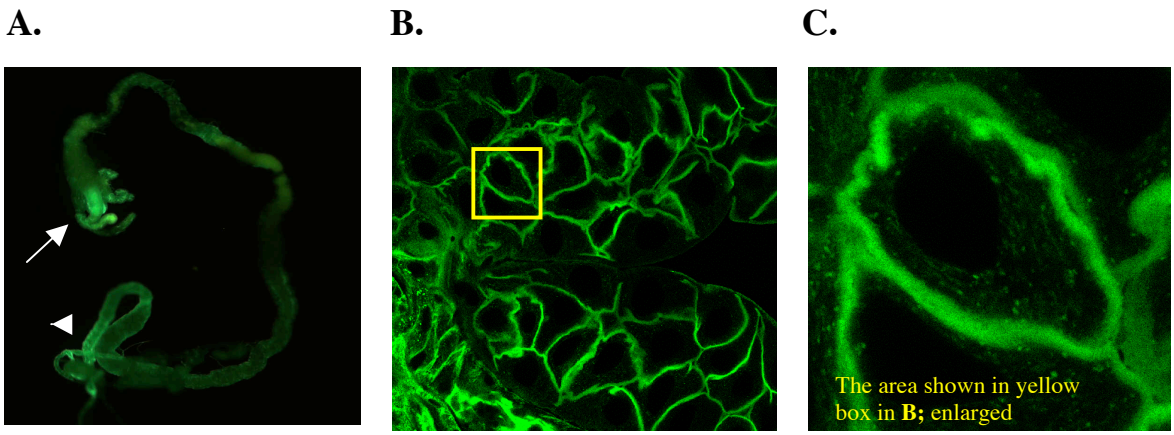


Fig. 7 ZnT-D1 localization. Pictures are taken from the larvae that overexpress ZnT-D1A driven by actin. **A.** Green fluorescence in the gut: note the accumulation of the protein in the proventriculus (arrow) and in the hindgut (arrowhead). 2.5x magnification. **B.** ZnT-D1 on the cell membranes (cells from the proventriculus region). 63x magnification. **C.** Enlarged cell shows nice green fluorescence on the membrane. 4x enlargement. ZnT-D1B-GFP fusion protein has similar localization.

Table 1

Genes downregulated at least two-fold in the MTF-1 KO larvae compared to the MTF-1 wildtype. P-value cutoff: 0.05. The last column indicates the number of MREs in the region 1 kb upstream from the annotated transcription start (or translation start, if the transcription start site is not known).

	genes (functions)	fold downregulation	MREs
1	MtnA, Metallothionein A	500	2
2	MtnC, Metallothionein C	143	5
3	CG4716	8.7	1
4	CG3994, zinc transporter-like	6.4	1
5	Ctr1B, copper transporter	5.7	4
6	CG6283, triacylglycerol lipase	4.9	-
7	CG5966, triacylglycerol lipase	4	1
8	CG6910	4	1
9	CG18576	3.7	-
10	kuz, kuzbanian, metalloendopeptidase	3.4	-
11	CG4840	3.2	2
12	CG3264, alkaline phosphatase	3.15	-
13	Hsp67Bb, Heat shock protein 67Bb	2.9	6
14	Hsp23, Heat shock protein 23	2.9	1
15	Mlp84B, Muscle LIM protein at 84B	2.8	1
16	CG3672, structural protein	2.75	1
17	CG17752, organic cation transporter-like	2.7	-
18	usp, ultraspiracle, ecdysteroid hormone receptor	2.7	1
19	CG11916, transcription factor	2.6	2
20	CG1793, transcription factor	2.4	-
21	CG17191, lipase-like	2.4	1
22	Adf1, Adh transcription factor 1, general RNA pol II transcription factor	2.4	-
23	CG10073	2.3	-
24	CG10632, enzyme	2.3	1
25	CG18632	2.25	-
26	aay, astray, phosphoserine phosphatase-like	2.2	-
27	emb, embargoed, nuclear export signal receptor	2.2	1
28	CG6597	2.2	-
29	CG5555, zinc ion binding, ubiquitin-protein ligase activity	2.15	3
30	CG8160	2.15	-
31	CG16713	2.15	-
32	Pcp, Pupal cuticle protein, structural protein of pupal cuticle	2.1	-
33	CG10960, sugar transporter	2.1	-
34	Smox, Smad on X, transcription factor	2.1	-
35	CG14970	2.1	-
36	CG5493	2.1	-
37	CG18217	2	-
38	CG18609	2	-
39	egh, egghead	2	1
40	cad, caudal, RNA polymerase II transcription factor	2	-
41	CG7093	2	-
42	FBMcm7, Minichromosome maintenance 7, DNA replication licensing factor 7	2	-

Table 2

Genes upregulated at least two-fold in the MTF-1 KO larvae compared to the MTF-1 wildtype. P-value cutoff: 0.05.

	genes (functions)	fold upregulation
1	GstD5, Glutathione transferase D5	5
2	CG13905	4
3	CG8588	3.3
4	alpha-Est7, alpha-Esterase-7	2.5
5	eIF5B, translation initiantion factor	2.4
6	CG6839, endonuclease	2.4
7	CG7381	2.15
8	CG13482	2.1
9	CG15784	2.1
10	CG7586	2.1

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RNA interference screen to identify the components of the metal response pathway in *Drosophila*

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Key words: metallothionein, copper, RNA interference

Abstract

Every organism has to cope with environmental changing amounts of transition metals, whereby toxic metals or an excess of essential metals have to be fenced off and a sufficient supply of essential metals has to be ensured. To maintain metal homeostasis, cells have evolved multiple mechanisms. The metal-responsive transcription factor-1 (MTF-1) is a key protein involved in the transcriptional regulation of metal homeostasis in higher metazoans, including insects, fish and mammals. A number of MTF-1 target genes, notably metallothioneins, have been identified both in insects and mammals. However the factors leading to the MTF-1 mediated transcriptional regulation are largely unknown. Using genome-wide RNA intereference technique in a *Drosophila* cell culture system, we have identified several candidate genes involved in the regulation of metallothionein expression in *Drosophila*.

Introduction

Addition of the essential metals copper, zinc, or the toxic metal cadmium to cells induces strong transcriptional response in *Drosophila* and higher eukaryotes. The metal-responsive transcription factor-1 (MTF-1), a zinc finger protein conserved from *Drosophila* to mammals, has been identified as an important mediator of this metal response. In mammals MTF-1 is required for viability; mice lacking the factor die from liver degeneration during embryogenesis (Gunes et al. 1998). In contrast, MTF-1 knockout flies survive. They are however they very sensitive to fluctuations of heavy metal concentrations (Egli et al. 2003). The experiments conducted in mammalian systems suggest that MTF-1 activity is regulated by phosphorylation and by nucleo-cytoplasmic trafficking (Smirnova et al. 2000; Saydam et al. 2001; Saydam et al. 2002). It has been suggested that multiple kinases and signal transduction cascades, including those mediated by protein kinase C, tyrosine kinase, and casein kinase II, are important for metal-inducible transcriptional activation of metallothioneins in mammals. It has also been found that MTF-1 interacts with nuclear factor κB (NF-κB) under hypoxic conditions to drive the expression of placenta growth factor (Cramer et al. 2005). Also, preliminary data in our laboratory suggest a possible interaction between MTF-1 and p300/CBP (Saydam and Schaffner, unpublished). Recently, a yeast two-hybrid interactome study suggested three interaction partners for *Drosophila* MTF-1 (Giot et al. 2003). Two of them, orthologs of *C. elegans* dumpy-30 protein, turned to be negative regulators of the MTF-1-mediated metallothionein expression in *Drosophila* (A. Vardanyan and W. Schaffner, unpublished). Also, it has been found recently that *Drosophila* MTF-1 regulates genes involved in both copper detoxification and acquisition by inducing the expression of metallothioneins and of a copper importer (*Ctr1B*), respectively (Selvaraj et al. 2005). One possible explanation of this dual behavior implies a hypothetical copper-dependent repressor protein that might regulate MTF-1 dependent expression in the *Ctr1B* locus. Taken together the data accumulated over recent years suggest several factors acting upstream or interacting with MTF-1 both in physiological and cell stress conditions to ensure a rapid and controlled transcriptional response. Even though several downstream targets of MTF-1 have been identified, factors upstream and factors cooperating with MTF-1 are largely unknown.

To identify genes involved in the metal sensing and further converting the signal into transcriptional activation of immediate metal-response genes, we took advantage of a high-throughput, full-genome screening method in the cell culture system based on RNA interference (RNAi). Several genome-wide dsRNA libraries have been constructed and successfully used in a number of organisms to selectively erase the cellular contributions of individual genes to study their function (Fraser et al. 2000; Gonczy et al. 2000; Berns et al. 2004; Boutros et al. 2004; Kittler et al. 2004; Paddison et al. 2004; Armknecht et al. 2005), for review see (Dasgupta and Perrimon 2004; Matthews et al. 2005). We have used the dsRNA library from the Drosophila RNAi Screening Center (Harvard Medical School, Boston) (Boutros et al. 2004). For the construction of this library, annotation by the Berkeley Drosophila Genome Project (BDGP) as well as an alternative Heidelberg annotation were considered (Hild et al. 2003). For our screening of metal response genes we chose the promoter of metallothionein A (*MtnA*) gene. *MtnA* is a well-established target gene of MTF-1 with a high basal expression and impressive induction upon heavy metal load both in vivo and in the cultured *Drosophila* S2 cells (Bunch et al. 1988; Zhang et al. 2001; Yepiskoposyan et al. in preparation). The high basal expression and robust induction are good prerequisites to screen for suppressors and enhancers of gene expression. In this study we tested the activity of the *MtnA* promoter by knocking down each of the 16,000 *Drosophila* genes individually in the normal cell culture conditions and upon copper load. Furthermore, we tested the candidate genes in the second, customized RNA interference screen. More than forty genes appear to act upstream of the *MtnA* promoter, whereby the vast majority is needed for *MtnA* activation, rather than repression.

Results and Discussion

To conduct a genome-wide RNA interference screen in *Drosophila* cell culture, we generated an S2 cell line that carries a stably integrated reporter gene with firefly luciferase under the control of the *MtnA* promoter and a reference with Renilla luciferase under the tubulin promoter (Fig. 1). We checked the inducibility of the metallothionein promoter by addition of the heavy metals copper, cadmium, zinc, iron, chromium, silver and mercury to the medium. All but iron and chromium induced the *MtnA* promoter to different extent. This is in agreement with our previous data where cadmium, copper and zinc were found to be the best inducers (Zhang et al. 2001; Yepiskoposyan et al. in preparation). Next, we checked the responsiveness of the system by knocking down the *MTF-1* gene with a corresponding dsRNA. The expression and metal induction of *MtnA* was more than six-fold lower in the cells treated with *MTF-1* dsRNA compared to the ones treated with dsRNA of lacZ gene or untreated cells (Fig. 2).

Having established the metal-responsive reporter assay we started full-genome screening by knocking down each *Drosophila* gene with a corresponding dsRNA in a 384-well plate format. Altogether 21,000 dsRNAs against more than 16,000 *Drosophila* genes were assayed individually. *MtnA* promoter activity was recorded in normal medium, or medium supplemented with 500 microM copper for 24 hours. Each screen was conducted in duplicate and the absolute value of each plate was normalized against the average of the plate. We used relatively mild criteria to score for genes whose absence influences *MtnA* promoter activity state. Firstly, we set a 1.5-fold change as a threshold compared to the average of the plate. Secondly, we considered only genes where dsRNA showed a similar change in *MtnA* activity in at least two out of four analyzed plates (with and without copper addition). Screening according to these criteria yielded a great number of genes that seem to influence the *MtnA* promoter when knocked-down. In total, knocking-down of 868 genes had a negative effect and knocking-down of 380 genes had a positive effect on *MtnA* activity (table 1). A considerable number of transcription factors, kinases, signaling molecules, transporters, pre-mRNA splicing factors, endopeptidases, structural constituents of ribosomes as well as structural constituents of cytoskeleton appear to be positive regulators of *MtnA* promoter in our screen (table 1A). The latter group of the genes however could appear as a result of a possible up-regulation of the tubulin

promoter that has been used as a reference in the assay. The genes, whose elimination induces *MtnA*, are candidates for negative regulation. They include a number of specific and general transcription factors, kinases, phosphatases, several histone genes, and, curiously some endopeptidase inhibitors (table 1B).

Copper transporters have a clear effect on *MtnA* activity. For example, when the copper importer Ctr1A was knocked down, metallothionein promoter showed reduced activity in agreement with a low copper state in the cell. Conversely, when the copper exporter ATP7 was targeted, the metallothionein promoter was activated to counteract the intracellular copper excess.

Interestingly, we found several components of Jun kinase (JNK) signaling cascade in our screen. For example, the knock-down of the gene coding for HNT (Hindsight, called also Pebbled) induced *MtnA* 2-to-3 fold. HNT is a negative regulator of JNK signaling. It plays important roles in embryonic dorsal closure as well as in tracheal development and eye morphogenesis (Wilk et al. 2000; Reed et al. 2001; Pickup et al. 2002). Yet dsRNA of CG7177, a serine/threonine kinase in the JNK cascade, down-regulated the *MtnA* promoter more then two-fold. In this context it is worth mentioning recent findings that place the JNK cascade at the center of a signal transduction network that coordinates the induction of protective genes in response to oxidative challenge, including metallothioneins A, ferritin heavy chain homolog, glutathione S transferase D1 and heat shock protein 68 (Wang et al. 2003). We tested the expression of the *MtnA* gene in several fly strains mutant for or overexpressing the components of the Jun kinase signaling pathway in an S1 nuclease protection assay. We could not however see any changes in the *MtnA* mRNA abundance in the flies lacking or overexpressing the hemipterous gene (JNKK) at normal or oxidative stress conditions, or in the flies heterozygous for basket (JNK), misshapen (JNKKKK) or puckered (JNK phosphatase, negative regulator) genes (data not shown).

Next, to nail down the genes upstream of the *MtnA* promoter, we have chosen 150 genes for further screening (see Table 2). These include (i) genes that strongly modulated *MtnA* activity when knocked down and/or were changed in all four plates, (ii) genes that might have relevance in heavy metal metabolism or generally in the stress response, not necessarily changed in the primary screen. The secondary screen have been conducted in the 96-well format, in the normal medium or medium

supplemented with 50 microM copper, each condition in quadruplicates. Each plate contained a positive control (dsRNA of MTF-1 gene) and negative controls (dsRNAs of *okra* and *morgue* genes). The average expression value of the plate has been taken for per plate normalization. A 2-fold threshold change at least in three out of four plates (for each condition) was considered for the screening (Table 3). According to these criteria, 23 genes reduced *MtnA* activity when knocked down under normal cell growth conditions and 18 genes did so at 50 uM copper. The majority of these genes had shown similar effects also in the primary screen. Interestingly, dsRNA against three of the tested five COMMD (copper metabolism gene MURR1 domain) genes reduced *MtnA* 2-to-3 fold (Table 3A). COMMD proteins are homologs of MURR1 (Burstein et al. 2005). MURR1 was found to be a general inhibitor of NF-κB (Ganesh et al. 2003). Moreover, mutations in MURR1 are responsible for copper toxicosis in Bedlington terriers, an inbred canine strain (van De Sluis et al. 2002). Recently it was demonstrated that MURR1 directly interacts with copper transporter Wilson protein, which suggests MURR1 involvement in the hepatic biliary copper excretion pathway (Tao et al. 2003). It is possible that COMMD genes play a role in copper transport also in *Drosophila*. If so, the downregulation of *MtnA* in the absence of COMMD genes argues rather for a role in copper import or inhibition of copper export, which would differ from the effect in mutant dogs. Next, particularly interesting is the *malvolio* (*mvl*) gene that codes for *Drosophila* homologue of mammalian natural resistance-associated macrophage proteins (Rodrigues et al. 1995). Malvolio is a divalent metal transporter, mainly specific for iron transport. *Malvolio*'s absence downregulated MtnA promoter more than 2-fold. However, when we tested the *MtnA* expression in the *mvl* mutant larvae in the S1 nuclease protection assay, we could not detect significant changes in the endogenous metallothionein transcripts (data not shown). Similarly, the S1 assay did not show significant downregulation of metallothioneins in the *tungus* (*CG12969*) mutant and in the heterozygous mutant of *enhancer of bithorax* (*E(bx), CG17135*). It is possible that the regulation observed in the S2 cells differs from that in the whole larvae. Certainly, more experiments have to be done to address this issue. Interestingly, among the genes, that appear to be important for *MtnA* expression under copper load is the *Drosophila* ortholog of p300/CBP, *nejire* (*dCBP*) (Table 3B). *Nejire* dsRNA reduces *MtnA* activity also in normal medium about 1.8 fold, but it was not included in our list when the above-

mentioned selection criteria were used. However, in the primary screen also *nejire* dsRNA was found to down-regulate *MtnA*. It would be interesting to test the contribution of dCBP on the expression of *MtnA* and other MTF-1 target genes. Finally, we would like to point out yet another gene, coding for Lilliputian (Lilli). dsRNA of *lilli* reduced MtnA expression in all our screens. Lilli is a transcription factor of a broad specificity. At least 16 interacting partners have been identified for Lilli in *Drosophila*. To our knowledge, it has not yet been implicated in the heavy metal stress response.

Further, the single homolog of mammalian Menkes and Wilson disease genes, *ATP7 (CG1886*) reproducibly appeared in our screens as a suppressor of *MtnA* expression (Table 3C and D). Another suppressor seems to be a poorly characterized zinc-finger transcription factor CG9215.

These results should be validated and the metal response genes further characterized. Recent studies in our laboratory indicate that *Drosophila* metallothioneins have differential metal-binding abilities and metal specific inducibility (Egli et al. submitted). It would be interesting to test if the candidate genes from this study have similar effects on the activity of all four *Drosophila* metallothioneins and other dMTF-1 regulated genes. This would shed light on the MTF-1 metal-specificity and the fine-tuning of the transcriptional response to heavy metal challenge.

Figure 1

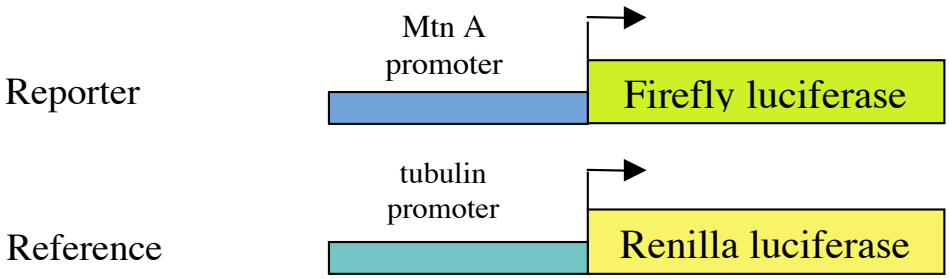

Fig.1 The schematic view of the luciferase reporter genes. The firefly luciferase reporter gene for MtnA and Renilla luciferase reporter gene for reference tubulin promoter are stably integrated into the genome of S2 cells.

Figure 2

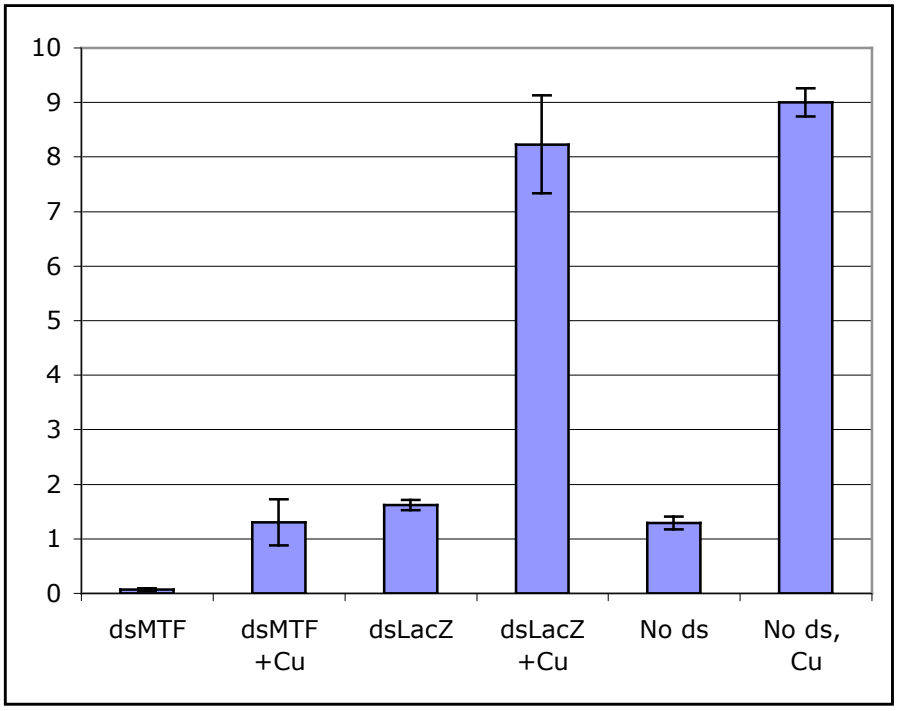

Fig.2 MtnA promoter activity in the S2 cells. dsRNA of MTF-1 downregulates both basal and copper-induced expression of MtnA-driven reporter gene. Cu = 24 hours 500uM $CuSO_4$ treatment, No ds = no dsRNA treatment

Table 1

A.

genes / gene families: dsRNA downregulates *MtnA*	
pre-mRNA splicing factor activity	25
specific transcriprion factor / transcription regulator	50
RNA polymerase	4
RNA polII transcription mediator activity / GTF / transcription	31
transcriptional elongation	3
translation initiation / elongation	9
translation repression	1
transcriptional (co)repressor	7
serine/threonine kinase, tyrosine kinase	23
pyruvate kinase, hexokinase	3
kinase inhibitor	1
phosphatase	7
phosphatase inhibitor	2
fatty acid biosynthesis/oxidation/metabolism, lipid, phospholipid, cholesterol metabolism	15
phosphate metabolism	1
monosaccharide metabolism	1
structural constituent of ribosome	51
cysteine desulfhydrase activity, transaminase	1
cation transport	15
anion transport	1
cytochrome-c oxidase	5
protein-nucleus import	1
(intracellular) protein transport	15
histone acetyltransferase activity, N-acetyltrasferase activity	3
ubiquitin-specific protease activity	3
ubiquitin conjugating enzyme, ligase	4
trypsin activity	2
exonuclease	1
membrane dipeptidase	1
metallopeptidase	3
endopeptidase / serine(aspartic)-type endopeptidase	28
procollagen C-endopeptidase	1
endopeptidase inhibitor	1
serine protease inhibitor	1
acyl-CoA metabolism	2
structural constituent of cytoskeleton	26
actin binding	2
polysaccharide metabolism	1
mitosis	1
cysteine-type peptidase	2
glucose transporter	1
mRNA cleavage	2
autophagic cell death	1
GTPase activator, small GTPase activity /intracellular protein transport etc.	15
RNA binding/mRNA processing	9
rRNA metabolism	1
nucleobase, nucleoside, nucleotide and na metabolism	5
signal recognition particle binding	1
ABC transporter/multi drug resistance	4
cyclin catabolism	1
glucuronosyltransferase	4
receptor, receptor binding, signal transduction	17
histone	1
structural constituent of larval/pupal cuticle	8
ubiquinol-cytochrome-c reductase activity	3
nucleic acid binding	1
pre-replicative complex formation and maintenance	1
DNA topoisomerase activity	3
defense response	5
aldehyde reductase	1

sphingomyelin phoshodiesterase activator	1
proline-tRNA ligase, tryptophan-tRNA ligase, arginine-tRNA ligase	3
taste receptor, perception of sound, visual perception, odorant binding	5
Hsp,Hsc	8
neuropeptide hormone, neuropeptide receptor	2
oxidoreductase/ electron carrier	4
amino-acid catabolism/metabolism	3
calcium transport / calcium ion binding/sensing	4
neurotransmitter secretion (t-SNARE activity)	2
ATPase activity	3
helicase	2
malate dehydrogenase, succinate dehydrogenase	2
fumarate hydratase	1
structural constituent of peritrophic membrane	2
carrier / transport	2
transketolase / pentose-phosphate shunt	1
ecdysone inducible gene, puparial glue	2
retinol dehydratase / aryl sulfotransferase	1
DNA repair, DNA replication, DNA recombination	2
hydrolase activity	2
caspase	1
mRNA localisation, catabolism	1
growth factor	2
carbohydrate metabolism/transport	1
peripheral nervous system development	1
proton transport	3
protein dimerization activity	1
SH3/SH2 adaptor protein activity	1
NADH dehydrogenase	1
structural constituent of nuclear pore	1
acyltransferase	2
mesoderm development, muscle contraction	1
Fer2LCH	1
carbonate dehydratase	2
transmission of nerve impulse	1
learning/ behavior/ memory/ mating	1
cathepsin	1
potassium channel	3
Ras interactor	1
tyrosine sulfotransferase	1
protein translocase	2
female gamete generation	1
cell adhesion	1
dipeptidyl-peptidase IV	2
allantoinase	1
glutathione transferase	1
chromatin assembly/disassembly	1
alpha glucosidase	1
Ctr1A, copper importer	1
nejire (dCBP)	1
MTF-1	1
unknown, protein dom.predicted/cell.comp./biol.proc. known	104
unknown	234
Sum	**868**

B.

genes / gene families: dsRNA upregulates MtnA	
eukaryotic initiation factor /translation factor	1
GTF	1
KDEL receptor	1
inositol-trisphosphate 3-kinase activity	1
nicotinic Acetylcholine Receptor / cation transport	1
ATP-dependent peptidase activity, metallopeptidase	2
specific transcription factor / transcription regulator	23
DNA repair, DNA replication	1
cyclin dependent kinase / ser/thr kinase	12
kinase, tyrosine kinase	3
ser/thr/tyr phosphatase	4
protein phosphatase / phosphoric monoester hydrolase	1
acyl-CoA metabolism	1
beta-N-acetylhexosaminidase activity	1
Ecdysone inducible gene	1
negative regulation of apoptosis	1
RNA binding	3
poly-A polymerase	1
oxidoreductase	5
intracellular protein transport / exocytosis	4
protein-nucleus export	1
ubiquitin-protein ligase activity	3
Fer1HCH 1/4	1
lipid/phospholipid metabolism, fatty acid biosynthesis/ metabolism	5
purine/ pyrimidine base metabolism	1
acetyltrasferase activity	1
histone	6
histone methylation	1
histone-specific chaperone	1
cation transport, organic cation transport	5
proton transport	4
malate dehydrogenase	1
structural constituent of cytoskeleton	8
carbohydrate metabolism /carbohydrate transport	4
ATP7 copper transporter	1
chromatin remodeling	1
protein myristoylation	1
oxidoreductase /electron carrier	3
ubiquitin-specific protease	2
small monomeric GTPase activity	7
ligase	1
glutathione transferase	1
glycosylation	3
receptor, receptor binding, signaling	10
protein phosphatase inhibitor	1
apoptosis induction	2
endopeptidase inhibitor	3
structural constituent of adult cuticle	1
nucleobase, nucleoside, nucleotide and na metabolism, trx reg.	2
Cyp, electron transporter	1
calcium binding / calmodulin binding, calcium transport	2
amino-acid catabolism/metabolism/transport	4
serine-type peptidase	1
female meiosis chromosome segregation	1
vitamin biosynthesis	2
long-chain fatty acid transporter	1
ABC transporter	3
translational initiation	1
helicase	1
tRNA metabolism (pseudouridilate synthase)	1
heparan sulfate sulfotransferase	1
nucleotidyltransferase	1
SH3/SH2 adaptor protein activity	1
growth factor	1
procollageb-proline 4-dioxygenase	1

endothelin-converting enzyme	1
hydroxybutyrate dehydrogenase	1
neuronal pentaxin receptor	1
peroxisome targeting signal receptor	1
actin binding/ brain development	1
metalloendopeptidase	1
NADH dehydrogenase	1
potassium channel	2
flotillin	1
olfactory receptor / perception of smell	1
innexin channel	1
defense response	1
acetylcholine biosynthesis	1
unknown, protein domain(s) predicted/cell.comp./biol.proc. known	66
unknown	128
Sum	**380**

Table 2

Position in RNAi Library **Plate 1**	Eurogentec name	Position in RNAi Library **Plate 1**	Eurogentec name	Position in RNAi Library **Plate 1**	Eurogentec name
A01	CG5034S1850	B10	vriS925	D06	srpS14264
A02	cadS3546	B11	Su(H)S2676	D07	srS14653
A03	CG3941R4411	B12	esgS2728	D08	CG7187R14670
A04	aptS4417	C01	CG17331R2916	D09	fruR14724
A05	Mef2R5645	C02	Dox-A2S3223	D10	bonS15094
A06	JraS5681	C03	CG18375R3930	D11	HLHm3S16163
A07	CG7022R7914	C04	CG5465S4121	D12	E(spl)S16169
A08	emcS8010	C05	Cdk9R4199	E01	Sox100BS17015
A09	dltS8208	C06	Mov34S4582	E02	ttkS17124
A10	CG11246S11743	C07	dpnR5289	E03	aseS17322
A11	RelR12958	C08	CG11979R5827	E04	CG15469S18027
A12	CG6572R14104	C09	BtbVIIS8424	E05	ovoS18041
B01	CG10278S14266	C10	CG12605R8568	E06	Rpt4R18258
B02	p53R15563	C11	CG5249S8846	E07	CG3032R18377
B03	CG17894R15590	C12	vvlS9054	E08	HiraS18459
B04	CG17741R15790	D01	ClkR9229	E09	l(1)10BbR19037
B05	l(3)mbtR16388	D02	CG7999S9289	E10	CG11695S19081
B06	kayS16771	D03	RpABC14S12241	E11	SmrR19255
B07	CG5113R18042	D04	Pros25S13611	E12	CG9215R19695
B08	nejR18778	D05	CG6118R14160	F01	BxS20236
B09	CG8924S19777				

Position in RNAi Library **Plate 2**	Eurogentec name	Position in RNAi Library **Plate 2**	Eurogentec name	Position in RNAi Library **Plate 2**	Eurogentec name
A1	CG8817S522	C7	CG17135R7915	F1	effR14027
A2	CG8222R1453	C8	Klp61FR8102	F2	CG7901S14662
A3	CG5853R1753	C9	CG16973R8329	F3	CG11460S14983
A4	bskR1821	C10	CG11591R8590	F4	Rab1R15218
A5	CG8193R5460	C11	CG4835S8823	F5	CG6892S15897
A6	JraS5681	C12	Pdp1R9232	F6	HLHm7S16168
A7	BEAF-32S6603	D1	CG6694R9367	F7	CG4963R16478
A8	CG14477S7077	D2	**MTF-1S9552**	F8	CG15504S16753
A9	BcR7207	D3	SodR9837	F9	l(1)scS17315
A10	CG7955S8165	D4	CG6100R9917	F10	uspR17596
A11	SnapS11454	D5	indR10501	F11	EG:100G10.7R17726
A12	CG7168R14678	D6	zetaCOPR10857	F12	pebS17987
B1	MvlR15177	D7	CG7580S10993	G1	CG12733S18080
B2	CG12106S18707	D8	CG14081R11230	G2	CG3977S18321
B3	CG2371R19093	D9	HLH106S11355	G3	ranR19006
B4	CG13780R1273	D10	IlkS11632	G4	CG1886S19125
B5	CG5356R1873	D11	CG7177R11703	G5	CG2577S19205
B6	CG6444S1951	D12	rnS12609	G6	CG10524S19217
B7	CG6043S2387	E1	CG2616S12685	G7	CkIalphaS19252
B8	fzyS2865	E2	pucS12762	G8	hepR19304
B9	DifR3004	E3	CG7459S12813	G9	Pp1-13CR19650
B10	Dox-A2S3223	E4	CG9603R12818	G10	CG8473S19743
B11	CG10505R3942	E5	CG9836R12919	G11	Pp2B-14DR19873
B12	CG15665S3949	E6	CG12952R12985	G12	B-H2S20014
C1	DebBR6083	E7	CG8454S12987	H1	B-H1S20023
C2	Mdr50S6509	E8	CG16750S13002	H2	fuS20245
C3	CG12969R6782	E9	CG9475R13092	H3	CG15455S20510
C4	catoR6879	E10	CoVaS13471	H4	CG1379R20520
C5	CG10404S7739	E11	CG6525S13604	H5	CG17167R20992
C6	rlS7869	E12	CG5844S13613		

Table 3

A.

	genes that reduce *MtnA* activity when knocked down, without copper	Plate 1	Plate 2	Plate 3	Plate 4	average	primary screen
1	CG3941; pita	-2.3179	-4.5027	-7.2466	-4.3379	-4.6013	down
2	apt; apontic	-1.2156	-2.4217	-8.4776	-5.6623	-4.4443	down
3	CG5853; ABC transporter	-2.8399	-4.9097	-3.0807	-6.3125	-4.2857	up
4	CG14477; muscle blind	-2.1047	-2.9457	-3.0491	-6.1406	-3.56	down
5	rlS7869; rolled	-2.3308	-1.1339	-2.4613	-7.8212	-3.4368	down
6	CG14081; MapK phosphatase	-1.8451	-4.9677	-2.5611	-3.0282	-3.1006	down
7	CG8193, tyrosinase	-2.9673	-1.2143	-4.5302	-3.6887	-3.1001	nc
8	CG13780, Pvf2	-3.7503	-2.2884	-3.1937	-3.0581	-3.0726	down
9	CG17135, E(bx)	-2.4496	-1.5188	-2.2446	-5.8459	-3.0147	up
10	cato; cousin of atonal	-2.3542	-1.6565	-2.0219	-4.7358	-2.6921	up
11	CG2371, COMMD10	-2.0788	-1.4541	-3.815	-2.9095	-2.5644	nc
12	Bc, Black Cell	-3.3326	-3.9219	1.40496	-4.3064	-2.539	nc
13	CG5356, COMMD5	-2.4887	-1.2468	-2.2686	-3.8901	-2.4735	nc
14	CG15665	-2.3935	-3.1181	-1.2964	-3.0005	-2.4521	down
15	Mdr50; multidrug resist. 50	-2.2396	-2.0598	-2.3302	-2.9056	-2.3838	down
16	Mvl; malvolio	-1.9177	-1.6052	-2.5044	-3.5045	-2.383	nc
17	CG6444	-2.1295	-1.7672	-3.033	-2.0098	-2.2349	nc
18	CG8454	-1.5072	-2.0876	-2.1553	-2.5767	-2.0817	down
19	CG8817; lilliputian	-2.3885	1.34271	-3.9927	-2.7686	-1.9518	down
20	CG7168; COMMD2	-3.3889	-3.8075	2.50752	-3.0789	-1.9419	nc
21	CG12969; tungus, endopeptidase	-2.3686	-2.119	-3.0956	1.1119	-1.6178	down
22	Ilk; integrin linked kinase	-2.8491	1.16094	-2.5131	-2.096	-1.5743	down
23	CG10505; ABC transporter	-2.9067	1.42535	-2.2529	-2.4082	-1.5356	nc

B.

	genes that reduce *MtnA* activity when knocked down, 50 uM copper	Plate 1	Plate 2	Plate 3	Plate 4	average	primary screen
1	Snap	-34.829	-1.6126	-6.2461	-9.8574	-13.136	down
2	CG11246; Rpb8	-2.5849	-3.8897	-3.5829	-26.643	-9.1751	down
3	Mov34; endopeptidase	-8.8632	-13.274	-2.2255	-2.6414	-6.751	down
4	HLH106	-10.985	-1.4578	-5.4276	-6.6372	-6.127	down
5	Dox-A2; endopeptidase	-9.2952	-9.641	-2.9687	-2.4923	-6.0993	down
6	zetaCOPR10857	-16.21	-1.6595	-2.7231	-3.0134	-5.9014	down
7	CG17331; endopeptidase	-6.7581	-7.2958	-3.4051	-2.5048	-4.9909	down
8	CG8222; Pvr	-6.6521	-1.2208	-3.7693	-4.9413	-4.1459	down
9	Rpt4; endopeptidase	-2.9491	-8.5232	-2.165	-2.7572	-4.0986	down
10	fzy; fizzy	-7.2802	-1.4103	-4.3822	-2.8576	-3.9826	down
11	DebB; splicing factor	-5.7072	-2.9486	-2.5431	-2.9	-3.5247	down
12	Klp61	-4.7362	-1.0731	-2.1581	-2.6826	-2.6625	nc
13	CG3977, Ctr1A	-4.0319	-1.0049	-2.3958	-3.1234	-2.639	down
14	CG7022; E(bx)	-3.5604	-1.1666	-2.8955	-2.8209	-2.6108	up
15	CG8817; lilliputian	-3.6231	1.5367	-5.1618	-2.4981	-2.4366	down
16	CG7999; Med24	-2.127	-2.9613	-2.32	-1.8083	-2.3042	down
17	CG6043, unknown	-2.7521	-1.3791	-2.0884	-2.2039	-2.1059	down
18	nejire, dCBP	-4.7568	-2.2503	1.27746	-2.0578	-1.9469	down

C.

	genes that induce *MtnA* activity when knocked down, without copper	Plate 1	Plate 2	Plate 3	Plate 4	average	primary screen
1	CG1886; ATP7, Cu exporter	12.9466	4.34246	4.19323	3.22216	6.17611	up

D.

	genes that induce *MtnA* activity when knocked down, 50 uM copper	Plate 1	Plate 2	Plate 3	Plate 4	average	primary screen
1	CG9215; txn factor	154.727	2.0888	-1.0341	3.39254	39.7936	up
2	CG1886; ATP7, Cu exporter	3.16277	1.78568	5.94193	5.50794	4.09958	up
3	fu; fused	1.86657	1.66871	4.1081	3.15621	2.6999	down
4	ase; asense	2.20197	2.78908	2.25163	1.21636	2.11476	down

Table 3. The genes that modulate MtnA promoter activity when knocked-down. The genes are arranged according to the fold-change of MtnA promoter activity. Only genes are shown where dsRNA results in more than 2-fold change in the *MtnA* promoter activity in at least three out of four plates. The column "average" shows the average fold change calculated for all four plates. We did not include here the genes that have high average fold-induction/downregulation, but are more than 2-fold changed in **less** than 3 plates. The last column shows if the given gene had an effect in the primary screen, nc = not changed. The color code here refers to the average expression of at least 2 independent wells where a similar change has occurred.

Color code:

2-3 fold downregulation
more than 3 fold downregulation
2-3 fold upregulation
more than 3 fold upregulation

Materials and Methods

Drosophila cell culture and stable transfection

Drosophila Schneider 2 (S2) cells were maintained in complete Drsosphila medium (Gibco Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (ICN Biomedicals GmbH) and 50 units penicillin G / 50ug streptomycin per milliliter at 25ºC. Transfection has been performed using the calcium phosphate technique. For subsequent selection of stable transfected cells, a selection vector that carries a hygromycin antibiotic resistance gene (pCoHYGRO) was co-transfected in 1:19 ratio with plasmids carrying the reporter (MtnA promoter fused to firefly luciferase coding sequence; pMtn-luc) and reference (tubulin promoter fused to Renilla luciferase coding sequence; pT-Renilla) genes. The selection was carried out in the medium containing 400ug/ml Hygromycin B.

dsRNA treatment

Double stranded RNA for MTF-1 and lacZ was prepared according to Kennerdell and Carthew (1998). The templates for in vitro transcription of dsRNA were generated by PCR using primers that are flanked by T7 polymerase binding sites at 5′ end. The dsRNAs were synthesized using T7 RNA polymerase (MEGAscript T7, Ambion) followed by DNase I digestion. The pilot screen was conducted as described bellow. The S2 cells used in all experiments carry stably integrated reporter genes for MtnA and tubulin expression. In the genome-wide RNA interference screen we used dsRNA library from *Drosophila* RNAi screening center (DRSC, Harvard Medical School, Boston) that contains 21,000 dsRNAs corresponding to each of the more than 16,000 genes in the *Drosophila melanogaster* genome (Boutros et al. 2004). Each dsRNA treatment was carried out in a separate well of 384-well plate. B2 well of each plate contained dsRNA of MTF-1 (positive control). First, 0,25ug dsRNA was incubated with $2x10^4$ cells with for 30 minutes in the serum-free medium. Following the incubation 3 volumes of complete medium were added to the cells which were then kept in the humidified chamber at 25ºC for three more days. On third day cells were $CuSO_4$ or mock treated. After 24 hours the firefly and Renilla luciferase was measured using Dual-Glo Luciferase Reporter Assay kit (Promega) and AnalystGT plate reader with stackers from Molecular Devices. For secondary screen, 96-well

plate format was used. The protocol was principally the same. Instead of 30 minutes, the incubation of the cells with dsRNA in serum-free medium was done overnight.

Data analysis

First, the absolute values of firefly luciferase measurement were divided by the Renilla luciferase values. The relative fold-change of firefly luciferase was calculated by dividing the values from each well by the average of the corresponding plate. The values of B2 well (dsRNA of MTF-1) as well as the standard deviation of each plate were taken into consideration.

Fly stocks

Animals were raised on standard cornmeal molasses-based food. For the oxidative stress experiment, paraquat was added to the food with a final concentration of 5mM. The fly stocks used in this study are:

- hemipterous mutant, hemizygous (w67c23 P{lacW}hepG0208/FM7c, Bloomington stock 12229)
- basket mutant, heterozygous (FRT40bsk^{1}/CyO, gift from P. Geuking and K. Basler)
- misshapen mutanat, heterozygous (FRT80msn^{10L}/TM6B, gift from P. Geuking and K. Basler)
- puckered mutant, heterozygous (pucE53/TM6B, gift from P. Geuking and K. Basler),
- UAS-hep (gift from P. Geuking and K. Basler)
- malvolio mutant (w1118; P{lacW}Mvl97f, Bloomington stock 5151)
- tungus mutant (w1118; P{PTT-GB}tunG00189, Bloomington stock 6838)
- Enhancer of bithorax, heterozygous mutant (P{ry11}E(bx)ry122, mwh1 ry506 e1/TM3, ryRK Sb1 Ser1, Bloomington stock 10628)

S1 nuclease protection assay

The nuclease S1 protection assay with 15, 20 or 30µg total RNA was performed as described (Weaver and Weissmann 1979). Total RNA was extracted using TRIzol reagent (Life Technologies). The dried gels were exposed to a storage phosphor screens and analyzed using PhosphorImager (Molecular Dynamics).

Acknowledgements

We are greateful to the *Drosophila* RNAi Screening Center for generous provision of dsRNA library and the screening facilities at the Harvard Medical School, Boston. We are thankful to Dr. Peder Zipperlen and Reto Städeli for the construction of the dsRNA library for the secondary screen. We are also thankful to Peter Geuking and Prof. Konrad Basler for kindly providing fly strains deficient or transgenic for the components of the JNK signaling pathway. We thank Bruno Schmid and Martina Mettler for the help in data ordering. This work was supported by Kanton Zürich and by the Swiss National Science Foundation.

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Discussion and Outlook

Essential metals are fundamental to many functions in the cells. The imbalances of these metals are known to cause several diseases. Hence the organisms developed mechanisms to maintain the balance of various metals. A central role in the homeostasis of heavy metals is played by metal-responsive transcription factor MTF-1, a zinc-finger protein conserved from insects to mammals. The MTF-1 mutant phenotype in mice and flies suggests several functions for MTF-1. In mouse it is essential for embryonic liver development and heavy metal detoxification. Although MTF-1 is not an essential protein in *Drosophila*, it is required for coping with excess metal, and, curiously, also with copper scarcity. Various aspects of MTF-1-mediated metal response have been studied, and several target genes, notably metallothioneins (MTs), have been established. Our studies identified novel MTF-1 target genes that, taken together, can explain all the known aspects of *Drosophila* MTF-1 function. MTs have long been suggested as a first line of defense against heavy metal load. The generation of a *Drosophila* MT "family knockout" firmly established their metal-defensive role and allowed for a deeper understanding of each family member's preference for different metals. Taken together, our data show that metallothionein mutants are highly sensitive to copper and cadmium load, but have only a marginal sensitivity to elevated zinc concentrations. Zinc exporter ZnT-D1 appears largely responsible for zinc detoxification. ZnT-D1 mutants are affected by the very same zinc concentrations where MTF-1 knockout flies suffer. In addition, the overexpression of ZnT-D1 results in zinc-super-resistant flies. And finally, the enigmatic low copper sensitivity of MTF-1 mutants was shown to be due to misregulation of the copper importer Ctr1B, yet another target gene of MTF-1. Unlike metallothioneins and other target genes, Ctr1B is induced by MTF-1 upon copper starvation (Fig.1).

Whereas many imetal-induced genes were identified in this and previous studies, the components of metal sensing and the metal stress transduction pathway are still largely unknown. Using RNA interference (RNAi) technique in cultured *Drosophila* cells, we uncovered a number of genes possibly involved in the metal response.

This study takes advantage of genome-wide screening techniques such as microarray-based transcriptome analysis and RNAi methods. These high-throughput techniques have proven to be far-reaching and fast methods in the discovery of new components of various pathways, and in our case, also of the metal-response pathway. However, since these methods yield large amounts of data, careful further tests are needed to examine the candidates. We selected several genes to test further in silico, in biochemical assays and genetically in the fly. These studies broadened our understanding on how a fly deals with fluctuating amounts of heavy metals. Our study not only discovered novel MTF-1 regulated genes, but also new aspects of well characterized genes; *Drosophila* ferritins, i.e., apart of having an important role in iron homeostasis, seem to take part in general metal detoxification. Interestingly, the latter, but not the first function, is MTF-1 dependent.

Anyhow, a good number of genes that emerged from our genome-wide screens still await further characterization. Some of them have established roles in other stress responses, and it will be interesting to test their contribution to metal homeostasis. However, large number of genes emerged from the screenings that are so far unknown. Many of them are evolutionarily well conserved and thus probably have important functions. This and similar studies are the first steps to reveal such functions.

Figure 1

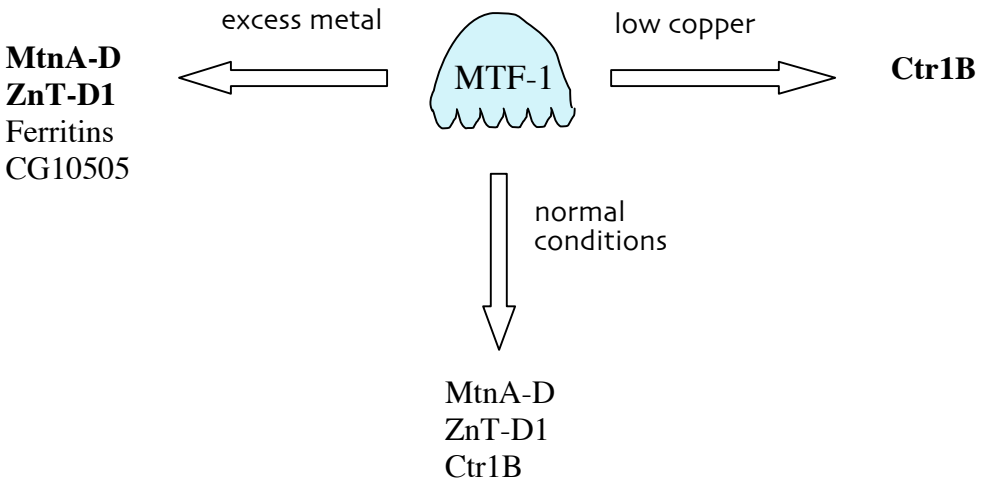

Fig.1 The role of MTF-1 in the metal homeostasis. At normal conditions MTF-1 ensures the basal expression of genes encoding all four metallothioneins (MT), zinc exporter ZnT-D1, and copper importer Ctr1B. Upon heavy metal load, *MT, ZnT-D1* expression is dramatically induced. In addition, MTF-1 upregulates several other genes, such as ferritins and ABC transporter *CG10505*. At low copper conditions the expression of Ctr1B is enhanced in an MTF-1-dependent manner. The genes in **bold** are induced by the indicated conditions in addition to the MTF-1-dependent basal expression.

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Acknowledgements

First and foremost I want to thank my supervisor Prof. Walter Schaffner for his constant support, guidance and encouragement at every stage of this thesis. The sharp-minded discussions led during our lab and institute meetings shaped me as a scientist and helped to enjoy the science a lot. Special thanks also to Dr. Erich Frei, Dr. Richard Eckner, Professors Markus Noll, Ernst Hafen, Konrad Basler, Peter Gallant, Michael Hengartner and Bernhard Dichtl from whom I learned a lot.

I am highly indebted to Dr. Oleg Georgiev for his colossal help during every day lab life. Oleg, dorogoi, I appreciate very much your friendship and help! Very special thanks to Toni, who is a great considerate friend for me.

It was a pleasure to work with Alla, Bo, Lidija, Till, Kappi, Carla, Iris, Andi, Ying, Mirjam, Ursula, Ivana, Gerhard, Martina, Michi, Simone, Bala, Rama, Haiqing, Mike, Hajime, Uschi, Alex and Bruno in the group. I want to thank Dieter for endless discussions and insightful directions that helped a lot in the course of all my work. No words can describe all support I got from Anand during all this time.

I wish to thank everyone in the institute of molecular biology and institute of zoology for an excellent research environment and support. Special thanks to Elena, Lilli and Kimon, Peter Geuking, Peder Zipperlen, Dani Bopp, Christof Hugentobler for his support in injections, Werner Wolz for IT support, Fritz Ochsenbein for preparation of figures, Werner Boll and of course, Michelle. I appreciate the quick and efficient service provided by the sequencing team, Titus Rauber as well as Irma and Maria.

I would like to acknowledge the collaboration we had with Prof. Dennis Thiele and Dr. Hao Zhou; Functional Genomics Center in Zurich, and especially the assistance provided by Andrea Patrignani and Dr. Ulrich Wagner; Drosophila RNAi Screening Center in the Harvard Medical School, Boston, and the great help from Dr. Susan Armknecht and Nadire Ramadan.

I should not forget my friends who helped me very much especially in the very early days of my (even not yet PhD) study: Sveta & Dima, Alexei. And of course all my armenian friends (Gayush, Roks, Sharkher, Goharik, Marina, Vahag, Lena, Aro, Garik, Narishok, Edo, Artak, Mara&Arman, Movses&family, Kara, Satenik, Natela, Tiko, Vahram, Artur and many others) for their endless support and love!

My dear friends Ruzok, Alla, Artash, also Taguhi, Arpi, Ani & Mika, thank you for being here next to me and sharing not only many troubles, but also much joy and frequent laughter.

I am most grateful to my sister, Sona, and to Hayk, who always stood by me and supported in all possible ways. Thanks a lot!

And last, but not least, thanks my parents, and especially my mother, who suffered my absence a lot and yet always encouraged all my commencements.